Diagnosis of Chagas Disease by Detection of *Trypanosoma cruzi* DNA Fragments from Filtered Urine Samples

by

Ana G. Madrigal

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Abstract

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*, is a potentially life threatening disease endemic to Latin America that has re-emerged as a global public health issue due to globalization. While this disease has been known of for hundreds of years, detection methods are still not straight forward; especially for the chronic phase of the disease. There is no golden standard for diagnosis and current detection methods rely on indirect tests such as serology; which has proven to be a suboptimal approach due to immunological and geographical variability.

Here, we report on a direct form of noninvasive diagnosis, focused on the detection of *T. cruzi*-derived cell-free DNA in the urine of Chagas Disease patients. For this study, samples were collected, stored, and processed using a filtration/extraction method. DNA was analyzed through conventional and nested PCR protocols, with three different primer sets targeting *T. cruzi* k-DNA or Sat-DNA repeat sections. The resulting positive PCR samples were confirmed through sequencing and compared against serological results. We found that *T. cruzi* DNA is indeed present in the urine of Chagas disease patients.

While further studies with a larger population are needed for improved primer development, the detection of *T. cruzi* DNA in urine has the potential to provide a novel non-invasive diagnostic method for Chagas disease that can be employed to improve chronic phase detection, treatment-monitoring, early detection in congenital disease cases and the management of immunosuppressed patient.

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I. Introduction

Chagas disease, a neglected tropical infection endemic to Latin America, has re-emerged as a global public health issue due to globalization and migration movements[1,2]; with approximately 8 million people infected worldwide and at least 100 million at risk of infection. This complex disease, caused by the protozoan parasite *Trypanosoma cruzi* (*T. cruzi*), consists of an acute and chronic phase that can cause heart and gastrointestinal issues depending on disease progression. While Chagas disease was formally described by Carlos Chagas 110 year ago (in 1909), there is still no single test for definitive diagnosis of infection with this protozoan pathogen. Detection of infection is complex, and the method used depends on the phase of the disease. For acute stages, identification of parasites in a blood smear is sufficient for definitive diagnosis. In cases of chronic disease when the parasite resides inside cells of somatic tissues, two serological analyses are recommended for a confirmatory diagnosis. However, serological tests have complications and are known to have regional variations in sensitivity[3,4] making diagnosis suboptimal and treatment-monitoring difficult[5]. To add to the diagnostic complications, the acute phase is often asymptomatic and detection in the chronic phase is hampered due to the parasite's concealed presence within host cells. These factors result in a serious under-diagnosis of *T. cruzi* infections in endemic and non-endemic areas. These diagnostic issues are also of importance in nonendemic areas, since health systems typically lack personnel with experience in recognizing and treating Chagas disease patients[1,6], especially in patients who present with reactivation or chronic disease.

It is also important to note that no specific treatments are currently available. Benznidazole and nifurtimox, which are the only current chemotherapy options, have

severe adverse effects and are not recommended for patients >50 years of age. In addition, these compounds are only effective against extracellular trypanosomes and not against intracellular amastigotes. As a result, cases of re-emergence of disease can occur. Also, chronic disease effects (such as cardiomyopathy, megacolon, and megaesophagus) require surgery or organ transplant, making early detection and speedy treatment of Chagas disease key for effective remedy.

In order to contend with these emerging issues, new strategies for diagnosis are needed. Different PCR strategies have emerged for identification of *T. cruzi*. Most focusing in DNA detection from blood samples, with primers targeting repetitive conserved DNA fragments found in either *T. cruzi* kinetoplast-DNA (k-DNA) or Satellite-DNA (Sat-DNA)[7–10]. So far, these new strategies are only used for serologically indeterminate samples, and screening tests with better sensitivities are being developed. Even then, these techniques work better for the acute phase, when there is a greater number of parasites in blood. In cases of chronic disease, the parasite is found in tissues and parasitemia levels are low, causing further complications for serological diagnosis.

This is why we have chosen to focus on an alternative diagnosis method that detects cell-free DNA in urine (also known as transrenal DNA or Tr-DNA) using PCR techniques targeting either *T. cruzi* k-DNA or Sat-DNA repeats. The detection of *T. cruzi* DNA in urine has the potential to provide a novel non-invasive diagnostic method for Chagas disease. PCR assays have previously demonstrated positive results days to weeks before circulating trypomastigotes are visible by microscopy of peripheral blood[11]. This may help improve chronic phase detection, treatment-monitoring, early detection in newborns from infected mothers (congenital disease) and manage immunosuppressed patients. The

utilization of filter paper for urine samples further facilitates sample collection, storage and transportation.

We hypothesis that cell-free *T. cruzi* DNA is accessible from the urine of patients as Tr-DNA and the PCR-based detection of *T. cruzi* Tr-DNA is a sensitive and specific measure of both the acute and chronic phases of Chagas disease.

In the following Chapters, I will describe Chagas Disease, going in depth into the parasite, its morphology and life cycle, what is known about its genetic makeup, the current worldwide view of Chagas disease and issues concerning its diagnosis.

Background

The Parasite

T. cruzi belongs to the genus *Trypanosoma*, a group of unicellular flagellate parasitic protozoa of the order Kinetoplastida. Trypanosomes are parasites that require both an insect vector and a vertebrate host in order to complete their natural life cycle. What differentiates *T. cruzi* from other members of the *Trypanosoma* genus is that they require an intracellular developmental stage in their vertebrate host and are mainly transmitted by the blood-feeding Triatomine bugs (commonly known as "kissing bugs", or "chinche").

Morphology & Lifecycle

T. cruzi changes its morphology throughout its different life cycle stages, going through three developmental forms: epimastigote, trypomastigote, and amastigote. A diagram of the life cycle by Pérez-Molina et al.[5] can be seen in **Figure 1**. The cycle starts when a reduviid vector acquires infection by ingesting circulating trypomastigotes from an infected host during a bloodmeal. Inside the vector, the trypomastigotes develop into epimastigotes, which replicate by binary fission in the insect midgut and differentiate into infective metacyclic trypomastigotes in the hindgut. The parasites are deposited within the vector's feces onto their mammalian host when the reduviid bug takes a blood meal. Infection is initiated when metacyclic trypomastigotes enter the bite wound site when the feces are rubbed or scratched onto the wound, or through mucosal membranes such as the eyes. Once inside the host, the metacyclic trypomastigotes penetrate any nucleated cell (with preference for muscle and epithelial cells, neurons, and reticuloendothelial cells)

and transform into amastigotes, again multiplying by binary fission and transforming into trypomastigotes. Once transformed they burst out of the cell and enter the bloodstream, where they can either penetrate another cell or be ingested by the vector[12].

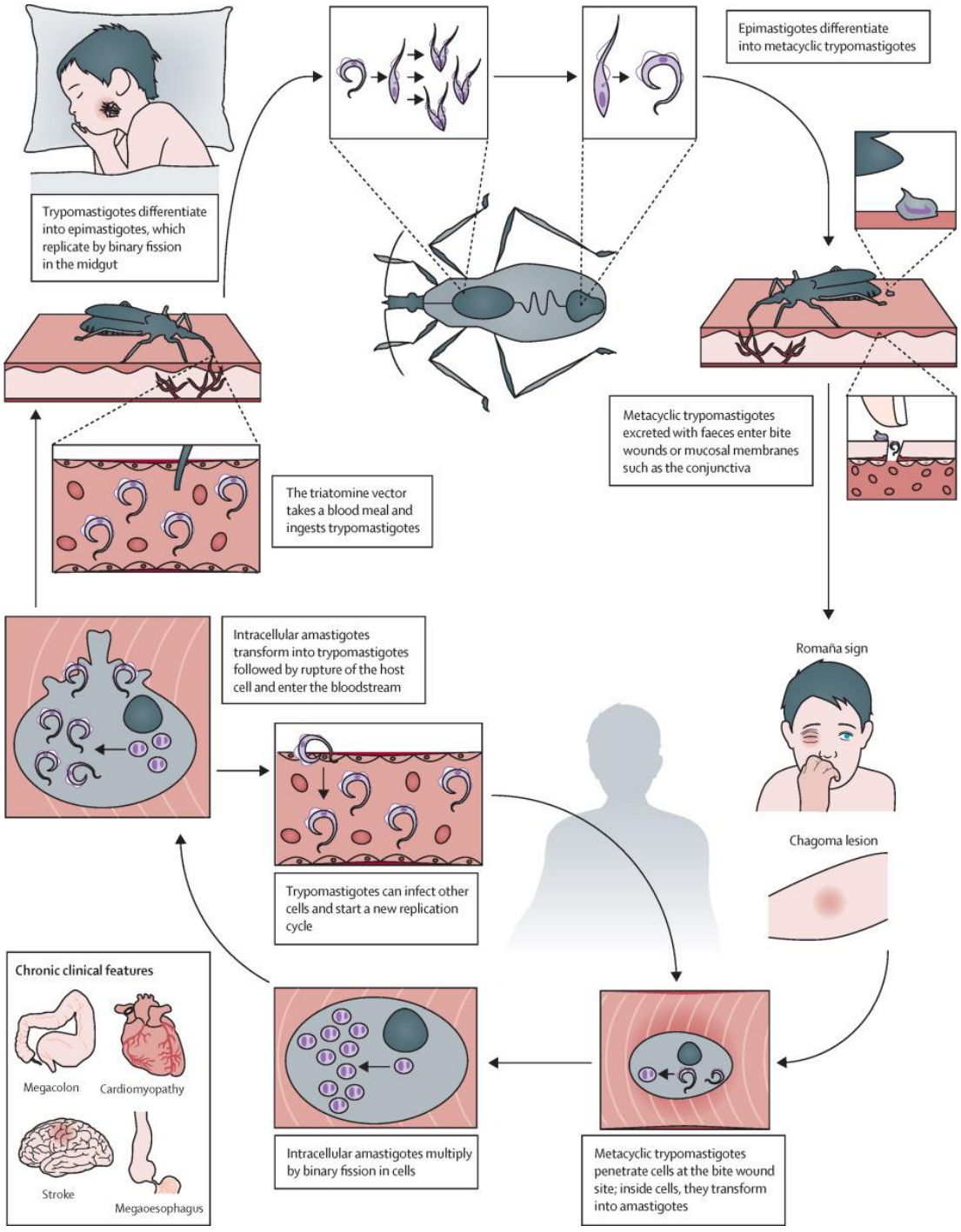

Figure 1) *T. cruzi* life cycle. Source: Chagas disease. Molina J, et al. The Lancet 2018; 391:1[70]0115-82.

The three forms of the parasite can be distinguished by the positions of their nucleus and kinetoplast (a network of circular DNAs representing many copies of the mitochondrial genome; see below) [Figure 2]. Trypomastigotes, found in the blood stream of mammalian hosts, average 20 μm in length and can take on either a long and slender morphology or a short and stumpy morphology[13]. These are the only forms of the parasite that are detected in Giemsa stained blood films, were they are generally seen in a C or U/S shape [**Figure 2**]. This form is distinguished by having a centrally positioned nucleus and a kinetoplast towards the posterior end, an undulating membrane and a flagellum running along the undulating membrane and extending from the body on the anterior end. The parasites also contain a flagellum that runs from the kinetoplast through the parasite's body and extends beyond it. Epimastigotes, found in the insect vector gut, are similar to trypomastigotes in general morphology but this time the kinetoplast is located anterior to the nucleus. Amastigotes, in the other hand, are found within the mammalian host tissue and are generally round (~2-4 μm) with an almost nonexistent flagellum.

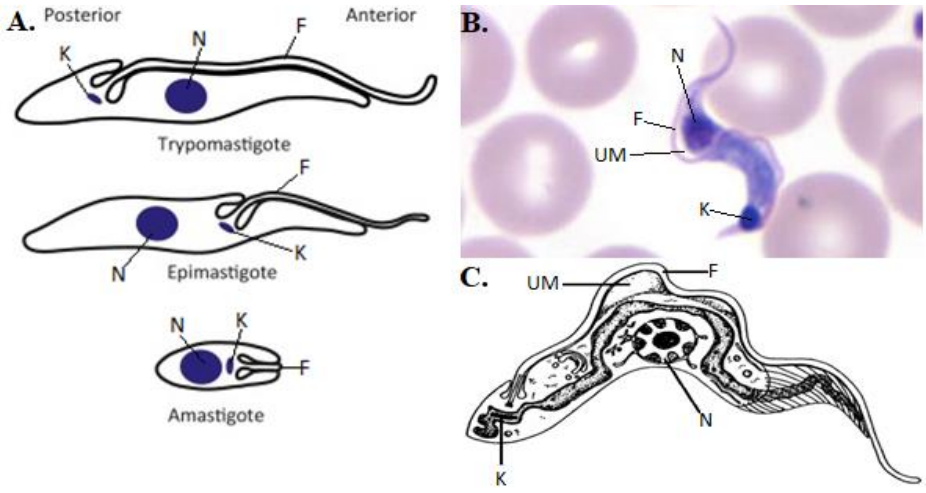

Figure 2) *T. cruzi* Morphology. **A.** T. cruzi morphology simplified cartoon adapted from Sunter, K=Kinetoplast, N=Nucleolus, F=Flagella, UM= Undulating Membrane. **B.** Trypomastigote in a thin blood smear stained with Giemsa, CDC. **C.** Morphology adapted from Souza 1999.

Phylogenetic Relationship

As mentioned above, *T. cruzi* belongs to the genus *Trypanosoma,* a genus that also encompasses other species that cause vector-borne diseases in humans; *Trypanosoma brucei* the etiological agent of Human African Trypanosomiasis (and another similar disease in livestock from related species); as well as *Leishmania* which causes different forms of leishmaniasis in humans. **Figure 3** contains a cladogram representing their consensus phylogeny[14] and was built based on MSP (Major Surface Proteases) gene family phylogeny rooted to *Ichthyobodo necator*. While the *T. cruzi* gene repertoire is broadly conserved in their chromosomal cores[14], their sub-telomeric regions demonstrated species-specific features; many of which encode cell surface proteins. While these regions help distinguish between the species, certain cell surface-associated protein families (such as δ-amastin expressed during the amastigote stage) are similar enough between *Leishmania* species and *Trypanosoma cruzi*[15] to result in cross reactivity (false positives) during certain serological, and rapid, diagnostic tests[14].

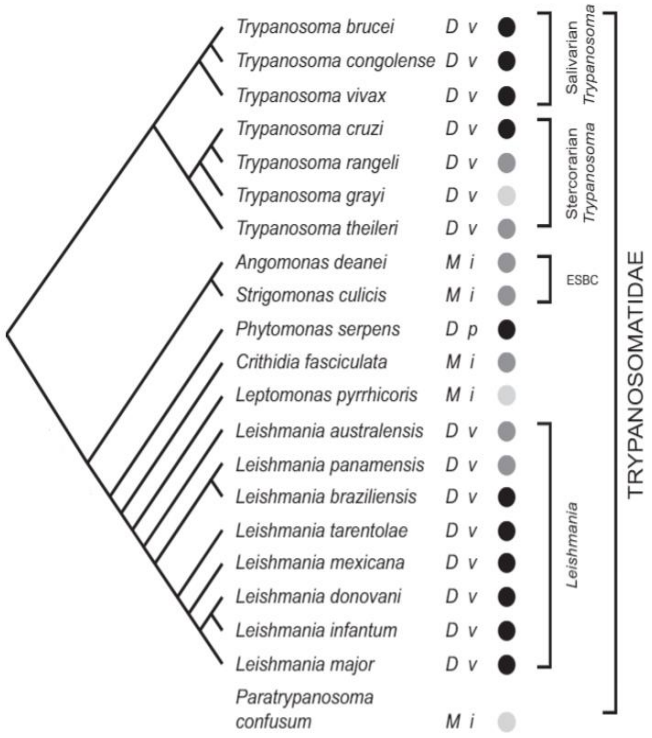

Figure 3) Trypanosomatidae phylogeny cladogram (edited from/adapted from Jackson, 2014). Note that this cladogram was built based on MSP gene family phylogeny and rooted to Ichthyobodo necator.

Genome

The *T. cruzi* genome was sequenced from a hybrid strain (CL Brener- TcVI) by the genome sequencing project[16]. However, its diversity and repetitive nature have made it difficult to completely assemble the linear sequence of its chromosomes. It is estimated that over 50% of the genome consists of repeated sequences[17,18]. Combined with its abundant level of diversity, the genome cannot be adequately defined by current genetic typing methods[19]. So far it has been found that *T. cruzi* is a diploid organism with homologous pairs of aneuploid chromosomes that vary in number and size among strains (varying up to 1.57-fold between strains)[16,19–22]. The mean nuclear genome size has been found to be 125 Mb (for TcII, TcV and TcVI isolates) and 89.5 Mb (for TcI isolates)[16]. This difference in sizes is in part attributed to a difference in number of repeat sections of DNA - as reported by Souza et al. – providing an explanation for the wide variation in genome size observed among eukaryotic species, which is more closely correlated with the amount of repetitive DNA than with the number of coding genes including retrotransposons and satellite DNA[16].

Satellite DNA (or Sat-DNA) is composed of very large arrays of tandemly repeated (a pattern of one or more nucleotides repeated directly adjacent to each other), non-coding DNA found in the centromeres. Sat-DNA was found to be 2.3 to 3.8 times more abundant in TcII, TcV and TcVI isolates than in TcI isolates[16], confirming that repeats do contribute to the difference in size of their genomes. A section of the nuclear mini-satellite region designated TCZ is present in 120,000 copies in the parasite genome, it is a repeated 195 bp sequence and represents 10 % of the parasites' total DNA [23].

Aside from nuclear-DNA, *T. cruzi* also contains a mitochondrial genome, known as the kinetoplast, that is composed of a complex of concatenated circles and minicircles. The minicircles represent 95% of the total kinetoplast-DNA (or k-DNA) and the whole mitochondrial genome represents about 20% of the parasite's total DNA[23]. K-DNA contains a high number of copies (10,000), and each minicircle is known to contain four regions of highly conserved DNA sequences (120 bp) between strains, and 4 regions of variable DNA (330 pb) [**Figure 4**][23,24]. The total amount of DNA (nuclear plus kinetoplast) ranges between 0.12 to 0.33 pg per cell depending on the strain and clone[16]. Since both Sat-DNA and k-DNA are present in multiple copies they can be used to our advantage in order to make diagnostic tests more sensitive. The most widely used PCR assays used for diagnostic of blood samples target either the k-DNA, or Sat-DNA repeat region[23].

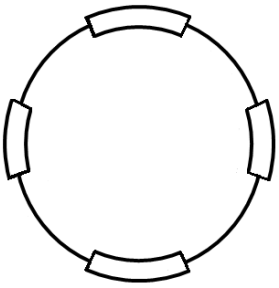

Figure 4) Representation of k-DNA minicircle 4 conserved (bold lines) and 4 variable (thin sections) domains.

Population Genetic Diversity

The wide genetic diversity of *T. cruzi* is currently classified into six genetic groups knowns as discrete typing units (DTUs), named TcI to TcVI, and a seventh unit called TcBat[25,26]. Several genetic molecular markers are used to classify the DTUs after their isolation in culture or directly from biological samples. The different DTUs present substantial genetic diversity with reports showing its effect on the parasite's biological, medical, and epidemiological characteristics[27].

A study[27] compiled a total of 6,343 published works to identify the DTUs of *T. cruzi* strains and their current diversity represents their overall distribution in the Americas [**Figure 5**]. TcI is predominant in the overall sample (around 60%), in both sylvatic and domestic cycles[27]. However, it is known that various DTUs can coexist in the same vector and in a single host[27,28], which could affect detection of overlapping DTU's in a single host.

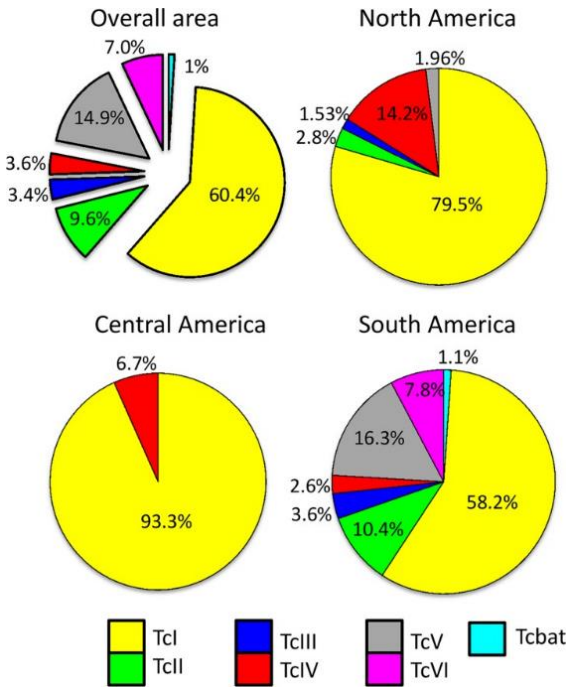

Figure 5) T. cruzi overall DTU distribution in the Americas. Source: Breniere et al, PLOS 2016; Breniere

Epidemiology

Epidemiology of Reduviid bug –

Figure 6) Triatomine bug, CDC[12].

In endemic regions, *T. cruzi*, is mainly transmitted by blood sucking Triatomine bugs, better known as "kissing bugs" (since they like to feed around the mouth of people). They are a subfamily from the Reduviidae, which is composed of mainly terrestrial ambush predators. They are easily recognized by their sturdy body, narrow neck, and their large curved proboscis[29], **Figure 6**. The parasite has a very broad range of insect hosts with more than 150 species of triatomines serving as an intermediate host. In turn, these bugs can transmit *T. cruzi* to over 100 species of mammals (reservoir hosts; such as armadillos, pigs, dogs, and cats) that maintain a large range of possible zoonotic sources to infect humans (definitive host). In the Americas, the range of the insects extends from the United states to Argentina and Chile with a total of 22 endemic countries[30].

Historically, the disease existed mainly in wild animals and rural areas. The disease was spread to domestic animals and to humans due to urbanization and rural exodus that has resulted in a shift in infection to urban or peri-urban areas[31].

Prevalence in Latin America–

Chagas disease has been a major public health problem in Latin American for many decades. Its area of endemicity includes countries such as Argentina, Brazil, Chile, El Salvador, Uruguay, and Mexico. Chagas disease is of main concern due to its

incapacitating effects and cardiac issues. In these endemic regions, the main mode of transmission is vector-borne. In 2010 Argentina, Brazil and Mexico were found to have the highest estimated number of *T. cruzi*-infected people (1,505,235; 1,156,821; and 876,458, respectively), Bolivia was found to have the highest estimated number of vectorial transmissions (8,087) followed by Mexico (6,135) and Colombia (5,274)[5,32].

With a number of measures for Chagas disease control setup up in the 1990s – such as vector control, improved housing programs, better detection programs, compulsory blood bank testing, and blood transfusion safety improvements – the incidence of *T. cruzi* infections has been reduced by an estimated 70%[5,33]. Prevalence has also decreased to the point of interrupting transmission for Uruguay in 1997, for Chile in 1999, and for most of Brazil in 2000[5]. The vector control program in Brazil has resulted in a documented savings in medical care and disabilities by US$17 for each dollar spent in prevention[33].

However, even with this progress, Chagas is still considered a threat. Since Chagas disease is not only vector-borne - it can also be transmitted through blood transfusions, organ transplantation, from mother to child (congenitally), or orally through contaminated food (only seen in the Amazon basin[34]. These and other issues, such as resistance to pyrethroid insecticides by the vector[35], have hampered disease control initiatives and increased frequency of transmission. Also, in South America, heart failure (which presents high mortality) is one of the main causes of hospitalization. It was estimated that the 10-year mortality rate for Chagas disease patients ranges from <10% to >80%, depending on the degree of cardiac damage[32,36]. In endemic areas of Chagas disease, infection is estimated to account for 41% of heart failure cases, and Chagas etiology may be responsible for 6.3% of these deaths[37].

Prevalence worldwide–

In 2010, due to political/economic reasons, migration from 17 countries endemic to Chagas disease was observed and Chagas disease became a global public health problem[38]. It has been estimated that over 8 million people are infected worldwide[39] [number changes to 10 million depending on the sources]; with an estimated 300,000 individuals infected in the United States, 80,000 in Europe and Western Pacific Region, 5,500 in Canada, 3,000 in Japan, and 1,500 in Australia[30,40]. **Figure 7** demonstrates the global distribution of Chagas disease in 2010. Estimates of the number of *T. cruzi*-infected individuals in non-endemic countries such as Australia, Canada, Spain, and the United States, were calculated by taking legal immigrant population from endemic countries and their prevalence of disease into consideration[40].

Figure 7) Global distribution of Chagas disease cases, based on official estimates, 2006-2010. Source: World Health Organization, 2010,WHO-Map[98].

Transmission in the U.S.A–

In the United States, *T. cruzi* infection is considered a neglected tropical disease, however, it has become a growing threat due to increased globalization. It is approximated that more than 300,000 (**Figure 8**) people in the United States are infected with *T. cruzi*, placing the U.S. second in prevalence after Latin America[10,41]. This estimate does not take into account undocumented immigrants and congenital infections. Possible vector transmissions in the U.S. has been monitored, especially in southern regions where 11 triatomine vectors species have been reported to maintain *T. cruzi* enzootic cycles[11]. These triatomines are present across the southern tier from the Pacific to Atlantic coasts. The greatest diversity of vector species was found in the southwest; particularly Texas, Arizona, and New Mexico; detected by the presence of *T. cruzi*-infected wild mammals[11].

Figure 8) Prevalence of Chagas disease imported cases in non-endemic countries. Source: Chagas disease. Rassi A, et al. [10] Lancet 2010; 375:1388-402.

To prevent transmission into the U.S., current FDA recommendations are to screen all blood donors using one of the two FDA-approved screening tests - blood testing is done through Radioimmunoprecipitation Assay (RIPA) to detect specific antibodies in serum[42]. Organ procurement organizations now perform selective or universal screening of donated organs for the presence of *T. cruzi*[42,43].

Animal reservoirs in the U.S.A. –

In the Western United States, woodrats are the most common reservoir but other rodents such as raccoons, skunks, and coyotes are also infected[11]. In the Eastern United States, raccoons, opossums, armadillos, and skunks are the most common reservoirs[11]. *Triatoma gerstaeckeri* is one of the most frequently collected and tested vector species in the United States[11]. The lower prevalence of Chagas disease in United States is attributed to the triatome species in this areas having delayed defecation (lowering transmission efficiency), as well as to the relatively better housing conditions that minimizes contact with the vector[44,45]. In total, seven autochthonous vector-borne human infections, five instances of organ-derived transmission from three donors, and five transfusion-associated *T. cruzi* infections had been documented in the States as of 2011[11]. All the transfusion-associated infections were associated with recipients who had underlying malignancies and were immunosuppressed[11].

Economic impact in the U.S.A.–

Chagas disease could also be an important economic impact in the U.S. An individual in the U.S. with chronic Chagas Disease is estimated to accrue an annual health-care cost of $2,161. This makes the U.S. second after Brazil in Chagas-disease-related

health care costs. At an estimated burden of 27,590 DALYs lost the U.S. is the second highest region for morbidity after Latin America[2].

Clinical Manifestations

Chagas disease has two main stages - acute and chronic.

Acute **–**The acute phase of infection can last between 6-8 weeks. While it is usually asymptomatic, some cases may present with malaise, enlarged liver/spleen/lymph nodes, prolonged fever, and subcutaneous oedema. At the site of the wound left by the insect vector, a chagoma may form if there is vector-borne transmission with *T. cruzi* entry through the skin. A chagoma is a reddened indurated area that may reach several centimeters in diameter and become very painful. The lesion typically subsides over a period of 2 to 3 months[13]. It is also known as a Romaña sign if it occurs in the ocular mucous membrane[10]. In the first weeks after infection, similar lesions to the initial chagoma may also appear elsewhere in the body[13].

Chronic **–** From these acute cases, ~70% never develop subsequent clinical manifestations. Presumably these patients are able to clear the infection. However, ~30% transition to the chronic phase of the disease that can persist for 10 to 30 years. The clinical manifestations diverge depending on the geographical area the disease is acquired, probably due to a difference in parasite strains[10,46]. The chronic phase ranges from asymptomatic (known as intermediate-chronic phase/ intermediate form) to severe illness and premature death. Manifestations include lesions of the autonomous nervous system in the heart, colon, esophagus, or the peripheral nervous system; cardiac alterations, digestive pathology, or neurological alterations[10]. When clinical manifestations are present they are divided into three major forms: cardiac, digestive and cardio-digestive[10]. The cardiac form

is the most serious and it accounts for 20-30% of the cases, making it the most frequent[47]. It includes heart abnormalities leading to cardiomyopathy, thromboembolism, cardiac failure, and sudden death. Typical heart abnormalities associated with Chagas disease are a combination of right bundle branch block and left anterior fascicular block, with heart failure more often related to late manifestations, and sudden death being the main cause of disease-mediated mortality[10].

The digestive form of Chagas is seen in 10-15% of chronic cases. This form is a progressive degeneration of the digestive tract leading to megaesophagus, megacolon, or both. The cardiodigestive form includes cases that have both the cardiac and digestive forms.

Around 10% of the infants from *T. cruzi*-infected mothers are born with congenital Chagas' disease[5,48]. Symptoms in congenitally infected infants include low Apgar score (health score for newborns standing for "Appearance, Pulse, Grimace, Activity, and Respiration"), fever, anemia, hepatosplenomegaly, hypotonicity, prematurity, and low birthweight[10]. Infection also associated with abortion and placentitis[10].

Congenital infection occurs in 1-10% of these infants and while most are also asymptomatic, a small portion cause severe morbidity presenting with anemia, hepatosplenomegaly, or respiratory insufficiency[49]. A more severe form of the disease is also observed in children younger than 5 years, and symptoms can include involvement of the central nervous system[13]. Immunocompromised infants have severe symptoms such as increased parasitemia (or infection reactivation in previously asymptomatic patients), severe myocarditis, and increased mortality. Cases of *T. cruzi*/HIV co-infections can also

lead to infection reactivation and sever neurological symptoms, often leading to misdiagnosis[13,31]. In people with HIV/AIDS, meningoencephalitis is the most frequent manifestation[50]. Also of note, since HIV-positive patients have weak antibody responses, serological diagnostics are not effective[31].

Immune response to T. cruzi

Acute – The immune response to *T. cruzi* infection is linked to the life cycle of the parasite. The consensus opinion is that *T. cruzi* emerging from infected cells and entering the bloodstream in high numbers is the trigger of the clinical manifestations observed during acute phase of infection. Antibodies recognize a spectrum of *T. cruzi* antigens. These antibodies are consistently present in infected subjects, with higher titers being observed in the acute phase[51,52]. The anti-*T. cruzi* antibodies are able to kill the extra cellular forms of the parasite by complement-mediated lysis[51] and in some individuals these antibodies are sufficient to eliminate the parasite[,52]. However, antibodies are not able to completely clear the intracellular stages of the parasite. An anti-*T. cruzi* CD8 T cell response has been identified, however, it does not appear to be strong enough to completely eliminate the parasite, but has been implicated in keeping the parasite under control[53]. Once inside muscle cells, heart tissue, the digestive system, or phagocytic cells, inflammatory lesions lead toward the chronic stage[7]. The parasite is able to evade the immune response by a combination of cell invasion, antioxidant enzymes, surface molecules and other compounds[53]. It has also been observed that the oxidative stress induced in infected macrophages, instead of being detrimental to parasite survival, favors persistence of the intracellular amastigote stage[53].

Chronic – The immune mechanisms associated with the chronic phase of infection is mostly unknown. It has been found that inflammatory lesions formed are dependent on CD4 T cells^{+}, CD8 T cells^{+}, IL-2, and IL-4; leading to heart failure, dilatation of the esophagus and colon[7]. It has also been proposed that this continuous inflammation causes an autoimmune-like disease due to cross-reactive epitopes that are common to molecules expressed by both *T. cruzi* and the host (also known as immune mimicry) [51].

Diagnosis

Diagnosis of *T. cruzi* depends on the phase of infection and traditionally relies on direct demonstration of trypomastigotes in blood or indirect detection of amastigotes in tissues with serological testing, hemoculture, or xenodiagnosis, and other methods.

Acute infection – The detection of the acute infection is based on the microscopic detection of trypomastigotes in blood. Microscopy techniques, including giemsa-stained thin and thick whole blood films or buffy coat preparations (microhematocrit technique), or serum precipitate (Strout technique) are used[5,10,32]. If positive for the acute phase, additional tests are used to determine whether the disease has entered the chronic phase.

Congenital – For congenital infections, diagnosis of the acute infection is based on the microscopic detection of trypomastigotes in blood through microhematocrit tests with cord blood or peripheral blood. Tests from neonates during the first month of life are recommended [10,54]. IgG serology at 6–9 months of age is also recommended when microhematocrit results are repetitively negative or not done early in life[10,55]. PCR has also been used for early detection by amplifying *T. cruzi* DNA in blood samples[10,56].

Chronic – Diagnosis of chronic *T. cruzi* infection is made after consideration of the patient's clinical findings as well as by the likelihood of being infected, such as having lived in an endemic country, or through additional tests after acute phase confirmation, such as an ECG (electrocardiogram), chest X-rays, echocardiograms, abdominal X-rays, and upper endoscopy[57]. For diagnostic confirmation, two different serological tests are required since no single, gold standard reference test is available[5,55,58]. During the chronic stage, trypomastigotes are usually not found circulating in blood thus serologic testing based on parasite-specific IgG antibody detection. Serological tests include Indirect

Immunofluorescence Assay (IFA), Indirect Haemagglutination Assay (IHA), or Enzyme-Linked Immunosorbent Assay (ELISA)[47,59]. These diagnostic tests are based on the presence of IgG against several *T. cruzi* recombinant antigens, synthetic peptides, whole parasite antigens or purified extracts[5]. PCR or immunostained Western blots may then be used as a third assay as backup for inconclusive serology results, especially when *Leishmania ssp*. might be present[46,60]. Parasites can also be detected in tissues by microscopy or PCR; however, this requires invasive techniques such as biopsies. **Table 1** lists the current available tests for Chagas disease detection cleared by the FDA[61] in the USA. Nonetheless, "The estimated index of underdiagnosis is still 95%"[5,62].

Blood Screening
ORTHO *T. cruzi* ELISA Test System
ABBOTT Prism Chagas
Diagnostic Tests
Chagatest EIA Recombinant
Hemagen Chagas kit
Lateral-flow test Chagas Detect Plus
ABBOTT ESA Chagas
Confirmatory Tests
RIPA
TESA-blot by the CDC

Table 1) List of tests available in the USA for Chagas Disease screening, diagnosis, and confirmation. *FDA*[61].

Each diagnostic method has its limitations. While serological tests are recommended for chronic disease detection, up to 3% of serology results can be discordant (with samples yielding inconclusive results in general clinical laboratories) [5]. In addition, serological tests are highly variable in sensitivity, especially between different geographical regions[4,63,64]. For example, certain tests show satisfactory results in regions such as Bolivia while the results from different populations such as Mexico and the United States are suboptimal[64]. These incongruencies may be due to differences in the antigens expressed by the multiple *T. cruzi* DTUs distributed in different endemic areas, variations in the human immune responses, and/or the use of different antigens between tests; for example, the use epimastigote antigens for diagnosis which could result in cross-reactivity

with epitopes found on molecules from other pathogens and possible false negative diagnostics[46,9,60,64]. Meanwhile, cultures and xenodiagnosis, though more affordable and proven to provide good sensitivity, can be inconvenient and test results take a long period of time to obtain.

Diagnosis takes time and effort, especially in non-endemic countries, where personnel are not trained to recognize the disease. A good summary of the process used in the detection chronic phase infection is presented by Molina et al. in **Figure 9**[5]: In summary, when a patient is suspected to have chronic infection (concluded from clinical history and symptoms) two serological analyses are required. When results are inconclusive, a third test is required from a reference laboratory for confirmatory results. Once confirmed, patients undergo a clinical evaluation (ECG examination, and echocardiography) to determine the phase of infection and recommended treatment follow-ups [5].

With current detection methods, there are also difficulties differentiating between newly acquired and past infections, since anti-*T. cruzi* IgG does not allow you to discriminate between an active infection or previous exposure to the parasite. Antibodies may still be circulating after successful treatment or undetectable, depending on tests used, method of sample collection, time after treatment, etc. [5,65].

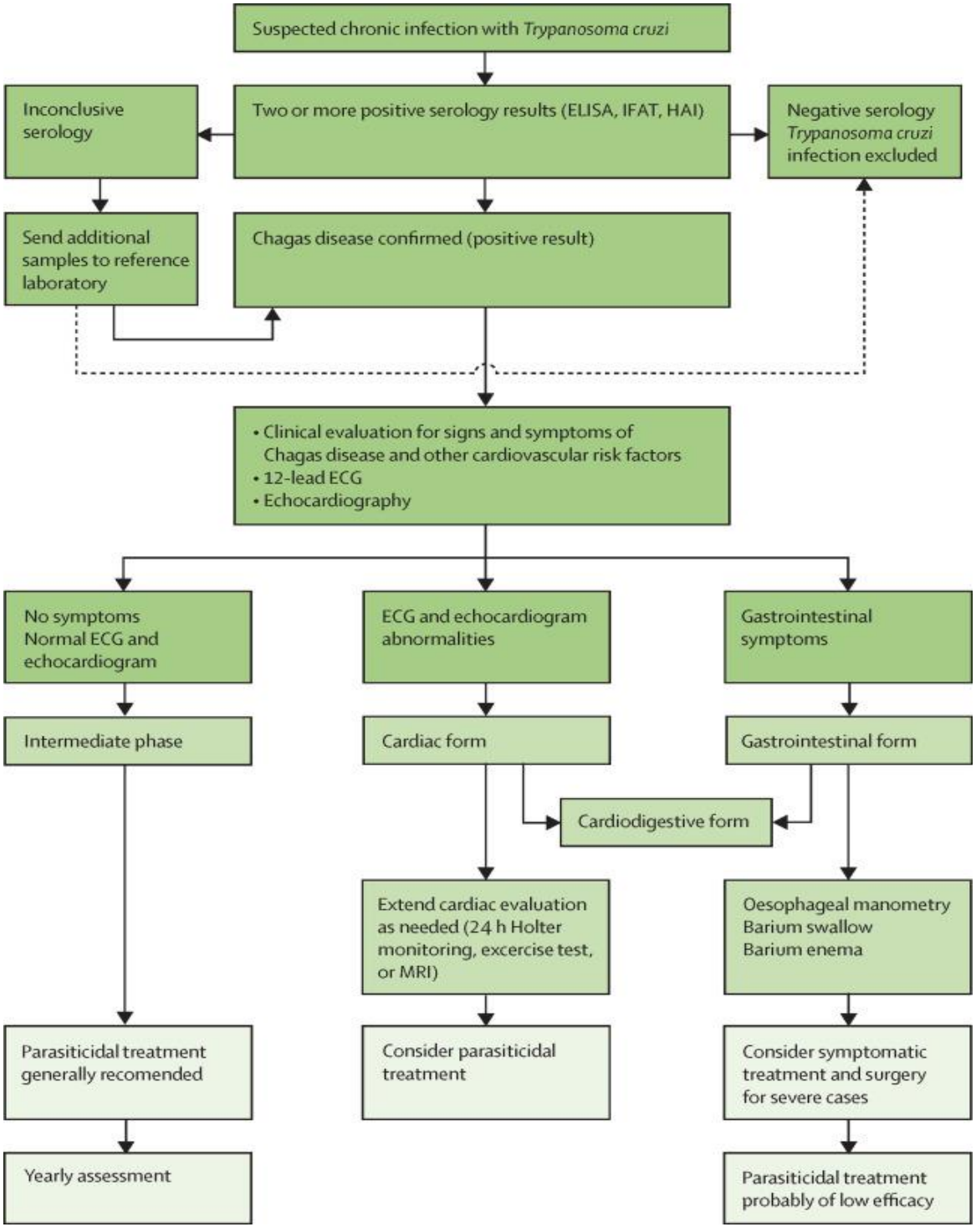

Figure 9) Chronic Chagas Disease Patient Assessment. Source: Chagas disease. Molina J, et al. The Lancet 2018; 391:10115-82[5].

Nucleic Acid-based detection of T. cruzi

An alternative diagnosis method is the detection of *T. cruzi* DNA as cell-free DNA (or cfDNA). Detection of cell-free DNA has potential for being a painless, convenient, non-invasive, and cost-effective approach that can be used in large-scale epidemiological screening programs[62]. While cfDNA detection is a relatively new concept for parasite diagnosis, it has a big potential of improvement and applications, and is already being used as an efficient biomarker in the fields of parental diagnosis (cell free fetal DNA or cffDNA), oncology (circulating tumor DNA or ctDNA), and as a method to identify fetal genetic disorders[62,66]. As the name suggests cfDNA are degraded DNA fragments found extracellularly mainly in circulation. While its origin is still unclear, it is thought to mainly originate from cells that undergo apoptosis[65]. It can also be found in urine, saliva, sputum, peritoneal fluid, synovial fluid, lymph, and amniotic fluid[,62]. The size of DNA fragments found in cfDNA range from 20 bp to 20 kbp depending on mechanism of release[67]. Cell-free DNA has been used to diagnose a number of human parasitic infections including *Plasmodium*, *Schistosoma*, *Trypanosoma*, *Wuchereria spp*, and *Leishmania*[62].

With advances in technology and techniques, new detection methods are arising. Such is the case of a quantitative real-time PCR (qPCR) study by Ramirez et al.[68] that targeted either Sat-DNA or k-DNA for a large number of blood samples from acute and chronic patients. While the clinical sensitivities of Sat-DNA and k-DNA assays were ~80%, both approaches were highly specific and reproducible. Also important to note is that PCR molecular techniques have been demonstrated to show positive results days to weeks before circulating trypomastigotes are visible by microscopy of peripheral blood[49]. Many other PCR strategies have also emerged for identification of *T. cruzi* DNA from

serum[7–10]. However, while these serum PCR-based assays have been found to be sufficiently specific, so far they are not commercially available and cannot be used for clinical diagnosis (due to sensitivity and performance variations); so far they are only used in cases where serological results are inconclusive [5,64,69,70]. Variations are caused by factors such as differences in DNA source (i.e. blood, tissue), collection, processing, sample volume, storage, parasitemia levels, and DTU variations; a more consistent protocol needs to be resolved and followed[69]. Also, this type of DNA-based molecular diagnostic method can be expensive and difficult to implement in endemic areas with limited infrastructure.

That is why we have focused our attention onto urine samples. A proportion of the cfDNA in the serum passes through the kidney glomerular barrier and is detectable in the urine by PCR amplification[71]. Two groups of cfDNA are found in urine: large fragments (>1000 bp) originating from cells of the lower urinary tract, and small fragments (around 150-250 bp) probably originating from circulation, also known as transrenal-DNA or Tr-DNA[72]. We selected filtered urine samples since previous work done in the Shiff lab, by Ibironke[73], Lodh[83] and Toribio[84], demonstrated that when urine is filtered, the DNA is stable on the filter for extended period of time. Ibironke and co-workers demonstrated this technique to be sensitive and specific in the detection of *Schistosoma haematobium* DNA – heretofore an infection only diagnosed through clinical assessment and microscopic detection of eggs in stool or urine (chronic and asymptomatic infections were often missed). The use of filters also facilitated specimen collection and transportation. Using a similar approach, detecting *T. cruzi* DNA through Tr-DNA PCR amplification presents a possibility for detection of Chagas disease in chronic phase, as well as early detection of

congenital disease, treatment-monitoring, inconclusive serology results elucidation, and management of immunosuppressed patients.

Treatment

Treatment of Chagas disease focuses on killing the parasite and managing the disease symptoms. Treatment with antitrypanosomal drugs is urgently recommended to patients that demonstrate the acute phase of the disease, congenital infection, and immunocompromised patients[74]. Drugs may also be offered to patients younger than 50 to slow disease progression, however, once the chronic phase is reached, medication alone is no longer curative and additional treatment is advised depending on symptoms (patients can be referred to cardiologists, gastroenterologist, or an infectious disease specialist)[13,74]. It has also been observed that effectiveness of treatment decreases with time from primary infection [5,75,10]. For heart related complications, treatment may include peacemakers, surgery, or heart transplant [57]. For digestive complications, diet modification, medication, corticosteroids, or surgery may serve as treatment[57]. For these reasons early detection and speedy treatment is key.

While no *T. cruzi*-specific treatments are currently available, two licensed antitrypanosomal drugs are available that have action on the blood forms of the parasite: Nifurtimox and benznidazole. Both are oral compounds that require prolonged administration and may display severe adverse effects, with safety and efficacy profiles far from ideal[5,76]. They cannot be used to treat pregnant women, are usually not recommended for patients over 50 years of age, and only show high efficacy when administered at onset of infection[10,77,78].

Nifurtimox was the first drug used against *T. cruzi*. It requires oral administration of 3-4 doses (8-10 mg/kg-adults; 15 mg/kg-children) throughout a 60-90-day period. Adverse effect frequency ranges from 43%-97.5%[5] including psychic alterations, digestive manifestations such as nausea or vomiting, anorexia, loss of weight, excitability, sleepiness, and occasionally intestinal colic and diarrhea[76]. Causing treatment to be discontinued in 14.5%-75% of cases[5].

Benznidazole is usually preferred since it is usually better tolerated by patients. It requires oral administration of 2-3 doses (5-10 mg/kg) throughout a 60-day period, with higher doses recommended in cases of meningoencephalitis[5]. Adverse effects include skin manifestations (e.g., hypersensitivity, dermatitis with cutaneous eruptions, generalized oedema, fever, lymphadenopathy, articular and muscular pain), with depression of bone marrow, thrombocytopenic purpura and agranulocytosis[76]. The drug shows evidence of neurotoxicity, ovarian toxicity, testicular damage, and harmful effects to colon, esophagus, adrenal, and mammary tissue[76] resulting in treatment to be discontinued in 9%-29% of cases[5]. It is the first drug approved (2017) in the U.S. by the FDA for *T. cruzi* infections (only approved for patients 2 to 12 years of age)[61].

Due to the adverse effects new treatments are required. These treatments also do not provide 100% clearance of parasite, they are effective against extracellular trypanosomes not against intracellular amastigotes, and cases of re-emergence of disease do occur[79,80].

II. Study Aims

We hypothesis that cell-free *T. cruzi* DNA is accessible from the urine of patients as Tr-DNA and the PCR-based detection of *T. cruzi* Tr-DNA is a sensitive and specific measure of both the acute and chronic phases of Chagas disease.

Rationale. As mentioned in the introduction, the current methods of PCR analyses focus on detection of *T. cruzi* DNA from serum, taking advantage of repeat genome sequences from Sat-DNA and k-DNA. We used a similar approach, however, this time adapting primers in an attempt to optimize *T. cruzi* Tr-DNA detection from urine samples.

We targeted the small repeat sections of *T. cruzi* Sat-DNA (195 bp; Tc Sat-DNA) and kDNA (conserved 124 bp and variable 330 bp; Tc k-DNA) that could be present in patient's urine as Tr-DNA (150-250 bp). kDNA is the *T. cruzi* mitochondrial genome that contains approximately 10,000 copies of a minicircle DNA. Each minicircle contains four regions of highly conserved DNA. For this reason, primers were selected to target different sections of *T. cruzi* kDNA: Primers 121-122 targeting the variable domain found between two conserved regions of the kDNA, and primers Minicon-1 and Minicon-2 targeting the conserved regions of the kDNA. Another set of primers was selected to target the Sat-DNA mini-satellite region, present in ~120,000 copies and representing 10% of the genome with a 195 bp repeat sequence. A nested PCR approach with primers TCZ1, TCZ2, TCZ3, and TCZ 4 was used.

III. Methods

In the following chapters, I will outline the process by which we tested our hypothesis. From sample collection and processing, to the DNA amplification and different protocols used.

Preliminary Studies

Study Population. Five samples, extracted from filtered urine, were obtained from an ongoing investigation on patients from Argentina with *Strongyloides* who were also suspected to being co-infected with *T. cruzi*. Four frozen urine samples, previously stored at -80°C, were provided by the Gilman lab (Johns Hopkins University, Bloomberg School of Public Health) as two ~40 mL samples collected from adults and two 5 mL samples collected from newborns of infected mothers.

Frozen Urine Extractions. To extract DNA from frozen urine, samples were first thawed at room temperature, after which they were filtered through 12.5 cm Whatman No. 3 (Whatman International, Maidstone, UK) filter paper and extracted following method described below.

The remaining two frozen urine samples were first thawed at room temperature after which Enk's[81] salting and resin extraction protocol was followed. Briefly, the two samples were adjusted to 40 nM EDTA, then heated at 100°C in a water bath for 10 minutes. After that, 5 *M* NaCl (in a volume of 1/10 of the sample volume) was added to each tube. The tubes were shaken vigorously for 15 seconds, placed on ice for 1 hour and centrifuged for 10 minutes at 4,000 rpm. The supernatant was transferred to another tube,

shaken vigorously for 15 sec and centrifuged for 10 minutes at 4,000 rpm. The supernatant was transferred to another clean tube, and absolute ethanol (two times the sample volume) was added. The DNA was then precipitated at -20°C for at least 2 hours. The DNA was removed with a pipette, transferred to a 0.5 mL microcentrifuge tube and washed in 200 µL 70% ethanol. The tubes were centrifuged again for 20 minutes at 14,000 rpm. The pellets were dried and suspended in 100 µL of DNAse free water and 100 µL of InstaGene matrix® resin (BioRad). Samples were incubated at 56°C for 30 minutes and 100°C for 8 minutes, vortexed at high speed for 10 seconds and centrifuged at 14,000 rpm for 3 minutes[81]. The supernatant was transferred to a new tube and DNA concentrations were measured using the Nano Drop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and were stored at -20°C.

DC Cohort Sample

Study Population. Forty urine specimens were collected from Chagas disease patients in the DC and Virginia area as part of a collaboration with Dr. Robert Gilman (Department of International Health, BSPH, JHU) and Dr. Rachel Marcus (MedStar Health & Vascular Institute). The individuals tested belonged to a cohort in a study titled "Chagas Disease and cardiac abnormalities in the Washington Metropolitan Area". Informed written consent was given for both sample collection and biorepository sample storage (consent forms were explained both in English and Spanish), samples were kept confidential with unique identification codes (IRB 00006713), and blind testing was followed to prevent bias.

Sample Filtration. All participants provided ~50 mL of urine in a plastic cup containing EDTA (for a final concentration of 40 mM, pH 8). Urine was then passed through 12.5 cm Whatman No. 3 (Whatman International, Maidstone, UK) filter paper, marked for subject identification and folded into a cone for easier filtration and concentration. The cones were mounted in single use falcon tubes to filter the urine at room temperature (**Figure 10**). After the urine had passed through the filter, filter discs were then unfolded and left to dry at room temperature before being packed with a desiccant onto individual plastic sleeves and stored at 4°C.

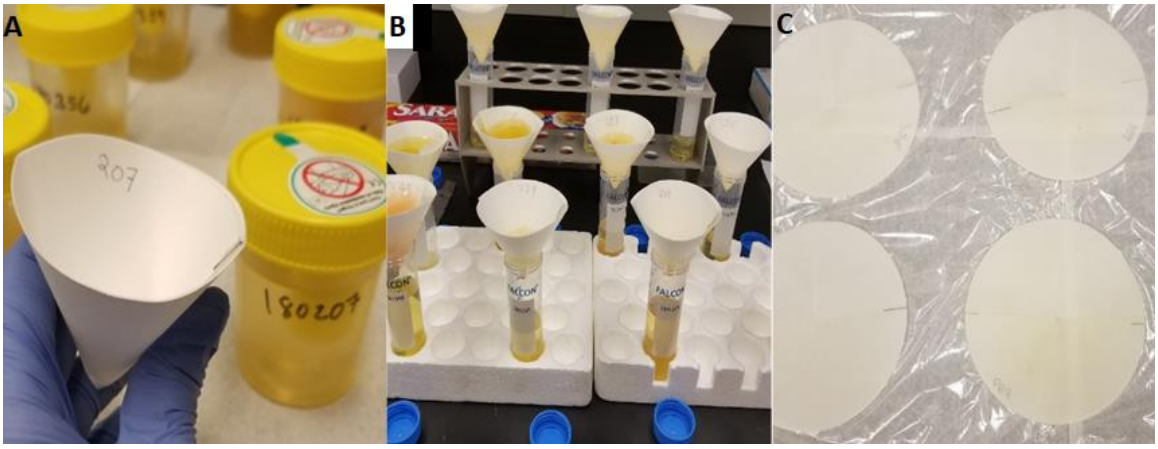

Figure 10) Filter urine process. **A.** Folded filter cone, **B.** Mounted filters, **C.** Drying.

DNA Extraction. To extract DNA from the filter paper, 10 punches (each 1/4") from the inner quadrant of the cone were taken (**Figure 11**). The paper puncher was cleaned with a 10% bleach solution and dried between each sample to avoid contamination. All 10 paper discs from a given sample were placed into a 1.5 mL Eppendorf tube and 400 µL of nuclease-free water was added. The tubes were then incubated at 95°C for 10 minutes and then mounted in a rotating water bath at room temperature overnight (~16-18 hours). The water-DNA solution was then transferred to Qiagen QIAmp 2 mL column, and DNA was

precipitated and concentrated using QIAmp DNA Blood Mini Kit (Qiagen, MD) following the manufacturer's protocol. DNA concentrations were measured using the Nano Drop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and were stored at -20°C.

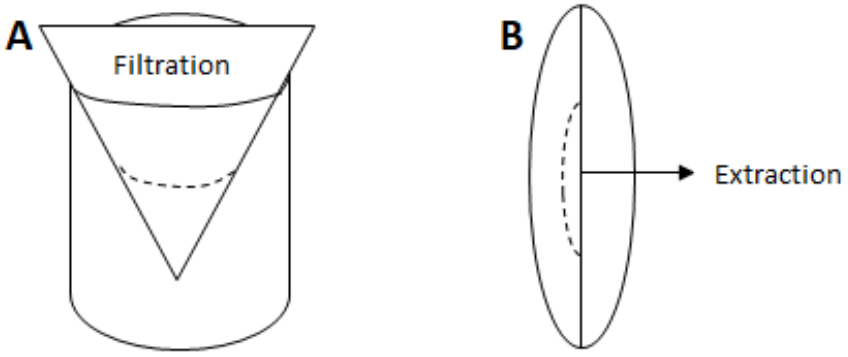

Figure 11) Filter placement. **A.** cone shaped filter with dashed line representing section in contact with urine were products accumulate.

B. Inner quadrant (dashed line) in contact with urine contains most of the concentrated DNA.

PCR Controls. *T. cruzi* genomic DNA (gDNA) was used as positive control. Genotyped as DTU II, ND, the control sample was provided by the Gilman group as a clot from a blood culture that was artificially infected with *T. cruzi*. To extract the DNA from the coagulate, the QIAmpDNA Mini Kit (Qiagen, MD) was used following the manufacturer's protocol for DNA purifications from tissues (steps 1-2c-3), and DNA purification from blood. DNA concentrations were measured using the Nano Drop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and were stored at -20°C. Urine samples obtained from individuals with clinical and serological negative results were used as negative controls. All reagents without DNA in each reaction were subject to amplification cycles as quality control.

Primer building. The 124 bp minirepeat reported for *T. cruzi* k-DNA (Degrave et al.[24]) was used as a query in BLAST using the non-redundant database of NCBI. Matches showing e-values < 0.001 and covering at least 90% of the query sequence were retained for further use. Based on this, k-DNA primers Minicon-1 (5'-TTT GGG AGG GGC GTT CAA AT-3') and Minicon-2 (5'-ACA CCA ACC CCA ATC GAA CC-3') were designed using PrimerBlast (PubMed). Primer properties and specificity for *T. cruzi* k-DNA were verified using BLAST search against non-redundant databases (PubMed, TriTryp).

PCR. All 40 DNA samples ranged in concentration ~1-3 ng/μL. 4 μL of sample DNA was used as PCR template. For the positive control, 1 μL of gDNA was used as template. Amplification was conducted in 0.2 mL PCR tubes in a thermal cycler (SimpliAmp™ Thermal Cycler, lifetechnologies™). Different primer sets were tested. See **Table 2** for a summary of primers used.

Primer		**Target**	**Amplicon**	**Reference**
121	5'-AAA TAA TGT ACG GGK GAG ATG CATGA-3'	k-DNA	330bp	Shijman[70]
122	5'-GGT TCG ATT GGG GTT GGT GTA ATA TA-3'			
Minicon-1	5'-TTT GGG AGG GGC GTT CAA AT-3'	k-DNA	113bp	This work
Minicon-2	5'-ACA CCA ACC CCA ATC GAA CC-3'			
TCZ1	5'-CCG ACG TCT TGC CCA CAC GGG TGCT-3'	Sat-DNA	149bp	Martins[82]
TCZ2	5'-CCT CCA AGC AGC GGA TAG TTC AGG-3'			
TCZ3	5'-TGC TGC AST CGG CTG ATC GTT TTC GA-3'			
TCZ4	5'-GAR GST TGT TTG GTG TCC AGT GTT GTGA-3'			

Table 2) Summary table of primers including target section of T. cruzi DNA, amplicon and reference.

In order to amplify the k-DNA variable domain, primers 121 (5'-AAA TAA TGT ACG GGK GAG ATG CATGA-3') and 122 (5'-GGT TCG ATT GGG GTT GGT GTA ATA TA-3') were used, generating a 330 bp fragment[70]. PCR protocol was adapted (from Shijman et al. [70]) for a total PCR reaction volume of 15 µL. Containing 7.5 µL of PCR Master Mix [2X, Promega, Madison, WI], 0.75 µL of each of the amplification primers (10 µM), 0.75 µL of 25 mM $MgCl_2$, and 3.25 µL of DNAs-free water to complete final volume. Thermal profile settings were:

94°C	3min	X1
97.5°C	1min	X2
64°C	2min	
94°C	1min	X37
62°C	1min	
72°C	10min	X1

Minicon-1 (5'-TTT GGG AGG GGC GTT CAA AT-3'), Minicon-2 (5'-ACA CCA ACC CCA ATC GAA CC-3') were used to amplify kDNA minirepeat section of the conserved domain, producing a 113 bp fragment. The PCR reaction was prepared to a final volume of 15 µL. Containing 7.5 µL of PCR Master Mix [2X, Promega, Madison, WI], 0.75 µL of each of the amplification primers (10 µM), 2 µL of 25 mM $MgCl_2$, and 2 µL of DNAs-free water to complete final volume. Thermal profile settings were:

94°C	10 min	X1
93°C	1min	X35
58°C	1min	
70°C	1min30s	
70°C	10min	X1

Nested-PCR (N-PCR) was used in order to amplify a 149 bp fragment from the Sat-DNA minirepeat domain[82]. Utilizing primers TCZ1 (5'-CCG ACG TCT TGC CCA CAC GGG TGCT-3') and TCZ2 (5'-CCT CCA AGC AGC GGA TAG TTC AGG-3') for the first reaction and TCZ3 (5'-TGC TGC AST CGG CTG ATC GTT TTC GA-3') and TCZ4 (5'-GAR GST TGT TTG GTG TCC AGT GTT GTGA-3') for the second reaction[82]. For the first step, the protocol was adapted (from Martin et al.[82]) for a total PCR reaction volume of 15 µL. Containing 7.5 µL of PCR Master Mix [2X, Promega, Madison, WI], 0.75 µL of each of the amplification primers (10 µM), 1.5 µL of 25 mM $MgCl_2$, and 2.5 µL of DNAs-free water to complete final volume. For the second step, 7 µL of previous PCR product was used as template for the second amplification, for a total final volume of 18 µL. Containing 7.5 µL of PCR Master Mix [2X, Promega, Madison, WI], 0.75 µL of each of the amplification primers (10 µM), 1.5 µL of 25 mM $MgCl_2$, and 2.5 µL of DNAs-free water to complete final volume. Thermal profile settings were:

Step 1		
95°C	5min	X1
95°C	30s	X5
60°C	30s	
72°C	1min	
95°C	30s	X25
65°C	30s	
72°C	1min	
70°C	10min	X1

Step 2		
94°C	5 min	X1
94°C	40s	X25
55°C	40s	
72°C	1min	X1
72°C	7min	

Analysis of PCR Products by Agarose Gel Electrophoresis. The PCR products were separated and sized on a 2% agarose gel. The gels were then post-stained with ethidium bromide solution (0.5 μg/mL) for 30 minutes, and de-stained in fresh distilled de-ionized water for 20 minutes with gentle agitation. The gel was then visualized with UV light. A ladder marker of 100 bp (New England BioLabs$_{Inc.}$) was used to estimate band sizes.

DNA Band Extraction and Sequencing. Resulting bands from positive samples were extracted from the agarose gel using the Monarch Nucleic Acid Purification kit (BioLabs$_{Inc.}$), following the manufacturer's protocol.

The eluted PCR DNA from positive samples were prepped for sequencing as recommended by Macrogen (Macrogen USA, MD), and sent separately (not premixed) with their respective primers to allow for sequencing conditions to be altered as needed. Results were then verified using BLAST search against non-redundant databases (GenBank, TriTryp). Positive samples were also run through a tape station (D1000 Screen Tape) to confirm the size of bands extracted (samples run and analyzed by Anne Jedlicka).

Bioinformatic analysis. To determine whether bands of different sizes found in the same sample were indeed fragments of repeats sections, samples were aligned and compared both to their corresponding positive controls and other bigger bands found in the same sample. Archived BLAST alignment results from samples were also aligned against the positive control using ClustalX with default settings.

Serology Samples. Serum samples were also collected from the patients who volunteered urine samples. The serum samples were processed and analyzed by Dr. Gilman's group

and the results of the tests were provided for comparison with the Tr-DNA results from each patient. A summary of the serology tests used can be seen in **Table 3**.

Test	Test Name	Approved	Description (Antibody/Antigen)
RT	Chagas Detect Plus	FDA	Immunochromatographic strip assay to detect anti-*T. cruzi* IgG. Only approved when coupled with a different serological test.
ELISA	Hemagen Chagas kit	FDA	Detection of anti-*T. cruzi* IgG. Lysate antigen of the epimastigote and amastigote forms of strain Y (DTU II) and CL (DTU VI)
ELISA	Chagatest EIA Recombinant v.3.0	FDA	Detection of anti-*T. cruzi* IgG that recognize the five recombinant antigens (SAPA, 1, 2, 13, 30, 36)
IFA		CDC	Detection of anti-*T. cruzi* IgG using a fixed preparation of epimastigotes. Time consuming, and has cross-reactive with Leishmaniasis
IHA	Chagas Polychaco Kit	CDC	Detection of anti-*T. cruzi* IgG against trypomastigote excretory-secretory antigens

Table 3) Summary of serology tests run. (RT- Rapid Test) (ELISA- enzyme-linked immunosorbent assay) (IFA- Immunofluorescent-Antibody Assay) (IHA- Indirect Hemagglutination Assay).

Statistical analysis. A Pearson correlation test, utilizing Graph Pad Prism, was done to determine if there was significant correlation between urine volume and DNA concentration. To illustrate consensus between tests run, Venn diagrams were constructed. Results were recorded for all tests and converted into numerical values (1=positive, 0=negative) for analysis of concordant pairs between tests.

IV. Results

Preliminary Tests and Optimization

Preliminary Sample Selection. To determine whether previously stored urine samples, at -80°C, could be used for PCR analysis, four frozen samples were provided by the Gilman group. Two samples (40 ml) were thawed, filtered, and DNA was extracted following the same protocol as the other filtered samples. The smaller two samples (5 mL) required a different method of DNA extraction. None of the four samples extracted demonstrated sufficient quality total DNA to perform PCR analysis. We concluded that freezing urine samples was not a proper method of preserving Tr-DNA.

For this reason, five filtered urine samples were provided by an ongoing investigation on patients from Argentina with *Strongyloides* who were suspected to being co-infected with *T. cruzi*. All samples and controls contained 1 ng/µL DNA concentration. These five filtered samples were used for preliminary and control runs.

Agarose Gel Electrophoresis Optimization. One of the issues encountered early on in our study was the ability to easily determine which samples were PCR positive by gel analysis. To enhance the ability to discriminate between positive and negative samples we revised the approach that we used for staining and visualizing the PCR products. The sensitivity between two different ethidium bromide (EtBr) protocols was compared to improve sensitivity (Figure 12). Both gels were prepped with 2% agarose and run with two water controls (W), two urine filtered samples (D-E), a negative control (-C), a positive control (+C), and 100 bp ladder for sizing. All samples and controls contained 1 ng/µL DNA

concentration. In gel A, EtBr (0.5 µg/mL) was included in the dissolved agarose during gel formation and allowed to set, after which samples were loaded and run (A, Figure 12). In gel B, samples were run through the agarose gel first, followed by a staining step with EtBr solution (0.5 µg/mL) for 30 minutes and de-stained procedure in water for 20 minutes with gentle agitation (B, **Figure 12**). The post-run staining method was demonstrated to be the more sensitive technique for visualizing the PCR results. The post-run staining protocol was used for all subsequent experiments.

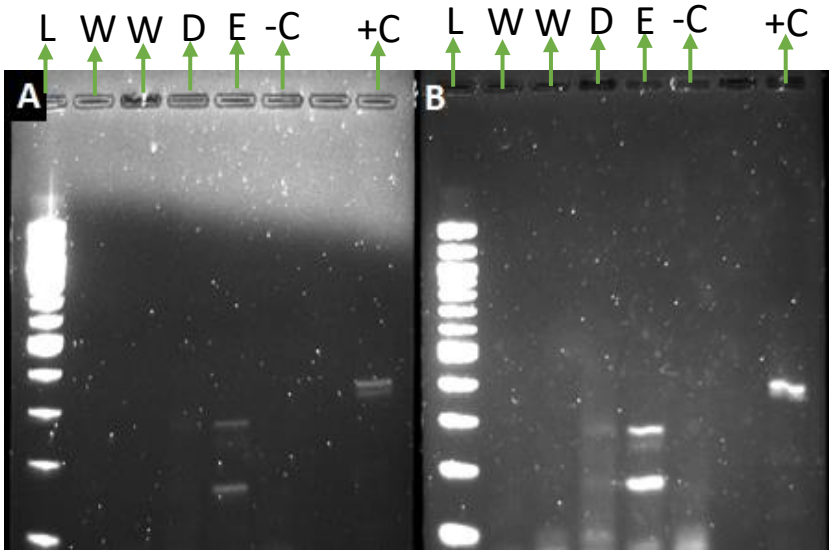

Figure 12) Comparison of 2% agarose gels EtBr staining protocols.

A. Pre-run staining

B. Post-run staining.

L= 100 bp Ladder, W=water control, D-E = urine filtered samples, -C= negative control, +C= positive control (gDNA).

Primer Selection. In order to determine whether existing primers (previously determined sufficiently specific and sensitive to detect *T. cruzi* DNA in serum samples) could be used to detect *T. cruzi* DNA from urine samples, five primer sets that produced small amplicons (<400 bp) were selected from different sources. Three of the primer sets targeted Sat-DNA and two targeted k-DNA (**Table 4**).

	Primer		Target	Amp.	Ref.
Conventional PCR	121 122	5'-AAA TAA TGT ACG GGK GAG ATG CATGA-3' 5'-GGT TCG ATT GGG GTT GGT GTA ATA TA-3'	k-DNA	330 bp	Shijman [70]
	P67 S34A	5'-TGGTTTTGGGAGGGGCGTTCAAATTT-3' 5'-TATATTACACCAACCCCAATCGAACC-3'	k-DNA	122 bp	Avila[99]
	cruzi-1 cruzi-2	5'-TGAATGGYGGGAGTCAGAG-3' 5'-ATTCCTCCAAGMAGCGGAT-3'	Sat-DNA	98 bp	Ramirez[100]
	TCZ-F TCZ-R	5'-GCTCTTGCCCACAMGGGTGC-3' 5'-CCAAGCAGCGGATAGTTCAGG-3'	Sat-DNA	182 bp	Shijman [70]
nPCR	TCZ1 TCZ2 TCZ3 TCZ4	5'-CCG ACG TCT TGC CCA CAC GGG TGCT-3' 5'-CCT CCA AGC AGC GGA TAG TTC AGG-3' 5'-TGC TGC AST CGG CTG ATC GTT TTC GA-3' 5'-GAR GST TGT TTG GTG TCC AGT GTT GTGA-3'	Sat-DNA	149 bp	Martins[82]

Table 4) Preliminary primers, target T. cruzi DNA, amplicon and reference obtained from. nPCR= nested PCR, Amp. =Amplicon, Ref. =Reference.

The five primer sets were compared following a control PCR protocol. The top performing primers were selected for further studies. Control PCR's were set up with two water controls (W), two urine filtered samples (D-E), a negative water control (-C), a positive control consisting of *T. cruzi* genomic DNA at 5.3 ng/µL (+C), and 100 bp ladder for sizing were run for each primer set, following thermal protocols as instructed by each of the primer sources.

Of the 5 primer sets tested, TCZ1-TCZ4 (nPCR) and 121-122 were the most sensitive (data not shown). The nested PCR (nPCR) targeting Sat-DNA resulted in the predicted band size of 149 bp in the genomic DNA (+C) and from one of the filtered urine samples (D, Figure 13), the other filtered sample was negative. Primers 121-122, which targets the k-DNA minicircle repeat, produced the correct band size of 330 bp for the *T. cruzi* gDNA positive control (+C), but different sized bands for one of the filtered urine samples (sample E, **Figure 13**), at 124 bp, 250 bp, and 270 bp. The 121-122 primer set did not amplify products from sample D.

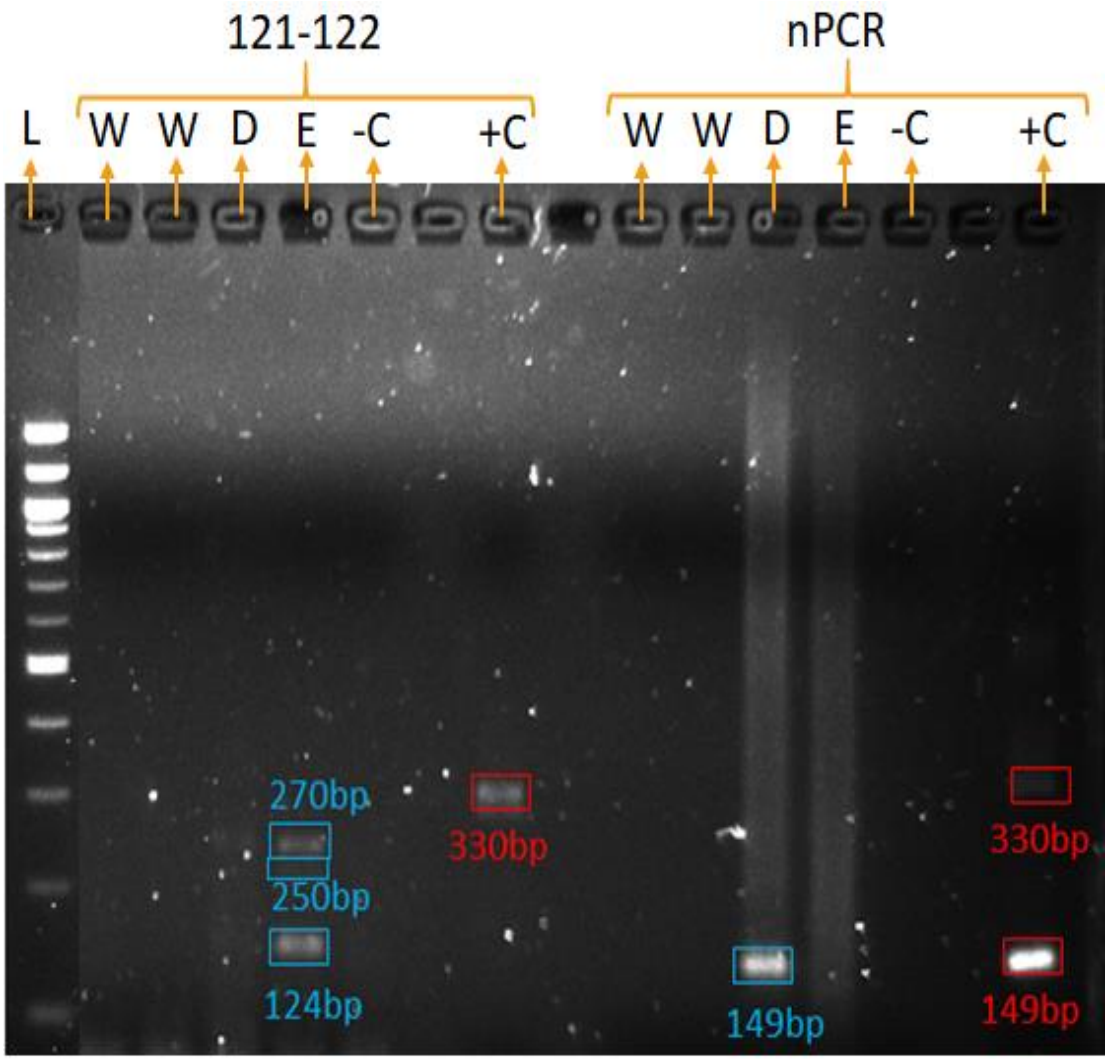

Figure 13) 2% agarose gel of primers: 121-122 and TCZ1-TCZ4: 121-122 (330bp) and nPCR (149 bp) L= 100 bp Ladder, W=water control, D-E = urine filtered samples, -C= negative control, +C= positive.

The thermal profile for primers 121-122 was optimized until clearer bands were observed and ran with the 5 filtered samples. Four out of the five samples were negative, with only sample E being positive. Still, the differently sized bands were observed in sample E (**Figure 14**) while the positive control (gDNA) demonstrated its corresponding primer amplicon band at 330 bp. Each of the different sized bands (124 bp and 270 bp) were individually extracted and sent to sequencing and identified using BLAST. These results demonstrated that, even though sample Tr-DNA and gDNA from the +C produced differently sized bands, primers 121-122 could still be used to detect *T. cruzi* k-DNA from filtered urine samples.

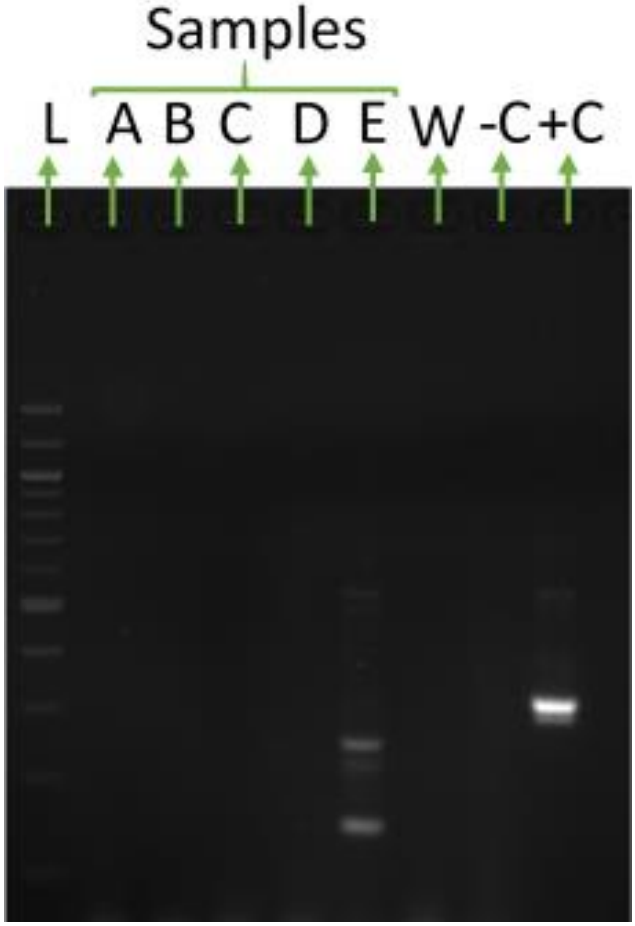

Figure 14) 2% agarose gel of optimized primers 121-122: (330bp) L= 100 bp Ladder, W=water control, A-E = urine filtered samples, -C= negative control, +C= positive control (gDNA).

New Primer building. While primers 121-122, targeting the minicircle variable domain, produced positive results, the bands observed from the filtered urine samples contained bands with sizes that were not predicated from the bioinformatics analysis of the minicircle sequences in databases. To get around this problem and obtain clearer results (with only one band per sample), we built a second pair of primers targeting the conserved domain of the minicircle (**Figure 15**).

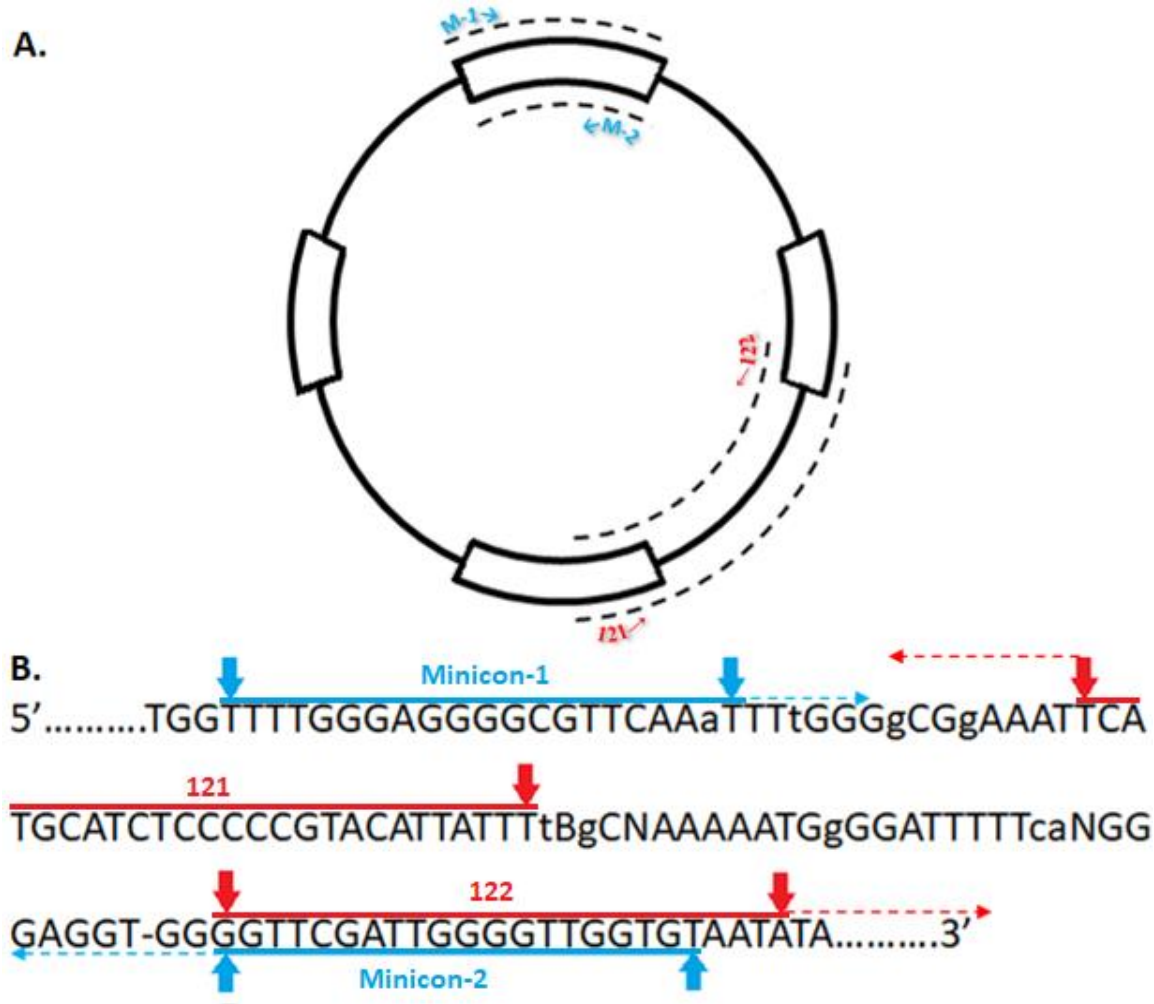

Figure 15) Representation of k-DNA minicircle with primer position. **A.** k-DNA minicircle with 4 conserved domain shown as bold triangles, and 4 variable domains as thin lines. Primer position and amplicon formation have been represented as dashed lines. **B.** DeGrave's minirepeat sequence demonstrating position and direction of primer sets 121-122 and Minicon-1-Minicon-2.

In **Figure 16**, results obtained with Minicon-1 and Minicon-2 primer set are presented. The primers produced the expected 113 bp band for the positive control. The predicted 113 bp band was also observed in the filtered urine samples, however two additional bands of ~120 bp and at ~300 bp were also observed. Each of the different sized bands (113 bp, 120 bp and 300 bp) were individually extracted and sent to sequencing. The resulting sequences were then identified as *T. cruzi* k-DNA using BLAST.

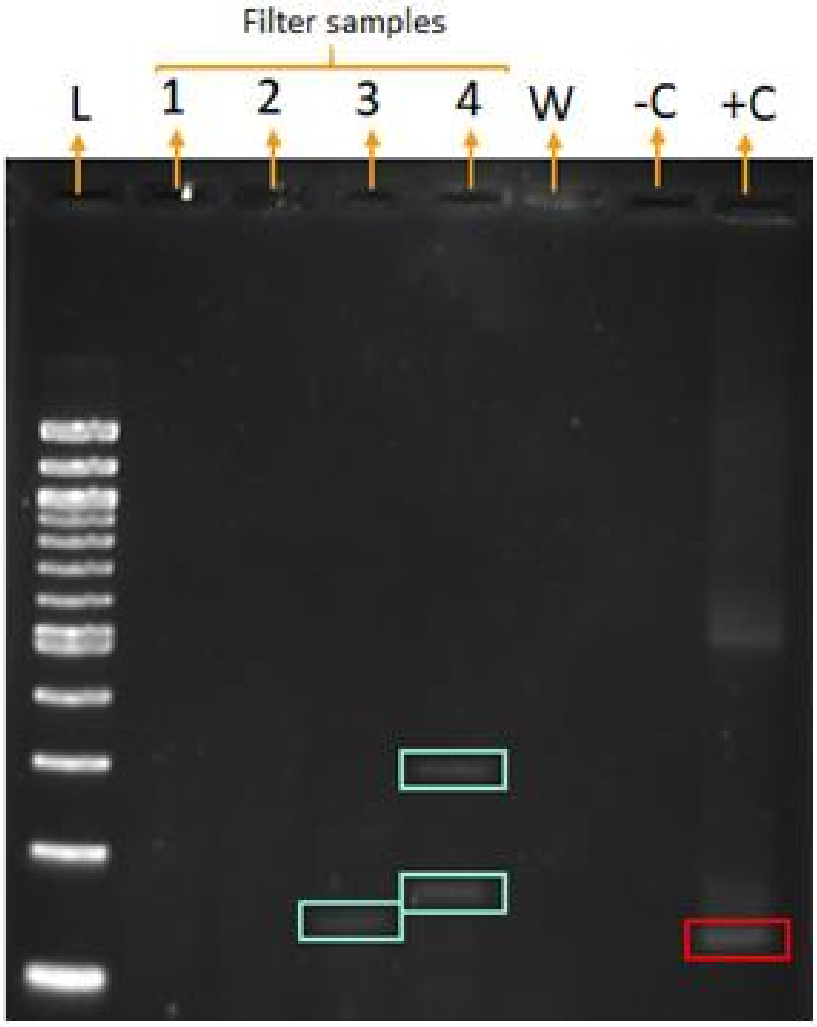

Figure 16) 2% agarose gel of primers: Minicon 1- 2. Demonstrating different band sizes at 113 bp, 120 bp, 300 bp. L= 100 bp Ladder, W=water control, 1-4 = urine filtered samples, -C= negative control, +C= positive control (gDNA).

Study primer selection: Primers for the nPCR (TCZ 1-4) produced the correct expected band of 149 bp (Figure 13), while primers 121-122 amplified two bands of different sizes (Figure 14) and Minicon-1-2 amplified one band of the expected size and two bands of bigger size than expected (Figure 16). While these bands were of unanticipated sizes, they were all confirmed as positive for *T. cruzi* repeat sections after sequencing. For this reason, all three primer sets were selected for further studies.

Table 5 contains a summary of all primer sets used for the study, including sequence, target, predicted amplicon size, and references). In the analysis of urine samples from the DC cohort outlined in the next section, we chose to test each urine sample with all three - 121-122, Minicon and nPCR - primer sets.

	k-DNA Primers		**Target**	**Amp.**	**Ref.**
cPCR	121 122	5'-AAA TAA TGT ACG GGK GAG ATG CATGA-3' 5'-GGT TCG ATT GGG GTT GGT GTA ATA TA-3'	k-DNA	330 bp	Shijman [70]
	M-1 M-2	5'-TTT GGG AGG GGC GTT CAA AT-3' 5'-ACA CCA ACC CCA ATC GAA CC-3'	k-DNA	113 bp	This article
nPCR	TCZ1 TCZ2 TCZ3 TCZ4	5'-CCG ACG TCT TGC CCA CAC GGG TGCT-3' 5'-CCT CCA AGC AGC GGA TAG TTC AGG-3' 5'-TGC TGC AST CGG CTG ATC GTT TTC GA-3' 5'-GAR GST TGT TTG GTG TCC AGT GTT GTGA-3'	Sat-DNA	149 bp	Martins [82]

Table 5) Summary of study primers targeting *T. cruzi*, their amplicon and reference. cPCR= conventional PCR, nPCR= nested PCR, M-1=Minicon-1, M-2=Minicon-2, Amp. =Amplicon, Ref. =Reference.

DC Cohort Samples Analysis.

Study participants. A total of 36 participants provided urine samples. Four individuals provided a second urine sample at a later time point, for a total of 40 urine samples. Of the 36 volunteers, 9 (25%) were male, 25 (69.4%) were female, and 2 (5.5%) preferred to not answer this question. While most of the participants (19; 52.8%) ranged between 40-59 years old (with a mean of 42.1 ± SD 11.8), 30.6% were in the 18-39 age group. The majority of the participants originated from El Salvador (18, 50%) or Bolivia (11, 30.6%). Summary of the demographics of the volunteers is presented in **Table 6**.

Variable	n	%
Sex		
Male	9	25
Female	25	69.4
NA	2	5.6
Age		
18-39	11	30.6
40-59	19	52.8
≥ 60	3	8.3
NA	3	8.3
Location		
El Salvador	18	50
Bolivia	11	30. 6
Argentina	2	5.6
Guatemala	2	5.6
Costa Rica	1	2.8
Peru	1	2.8
NA	1	2.8

Table 6) Epidemiological variables in study population (n=36).

Electrophoresis Results. **Figure 17** shows an example of the gel results using the same set of DNA templates for each of the three primer sets. Each PCR was run blindly to prevent bias. Following thermal cycling protocols outlined in the methods section, each gel was run with numbered filtered urine samples (1-10), a negative control (-C), a positive control (+C), and 100 bp ladder (L) for sizing [Results master table in the Appendix; Table 10].

From the three primer sets used, the nested PCR targeting Sat-DNA proved to be the most sensitive. Producing the predicted sized band of 149 bp in both the positive control (+C) and filtered urine samples (**Figure 17**-A).

Minicon primers, targeting k-DNA, produced the correct sized band of 113 bp for some samples, and different sized bands (one at ~120 bp, and a third at ~300 bp) for other urine samples. It also produced the correct sized band for the positive control (+C) at 113 bp (**Figure 17**-B).

Primers 121-122, targeting k-DNA, produced the correct band size of 330 bp for the positive control (+C) but bands ~124 bp and/or ~270 bp from the clinical samples (**Figure 17**-C). Previous sample from the Argentina preliminary results was also used as comparison against the newly acquired samples and a difference in sensitivity was observed (results in **Figure 25**; Appendix).

All bands, including each of the different sized bands, were individually extracted and sent for Sanger sequencing. All resulting sequences were then identified positively as *T. cruzi* k-DNA using BLAST. Thus, despite generating PCR products different from the predicted size, based on the literature and informatics analysis, all of the PCR products, regardless of size, were verified as originating from *T. cruzi* minicircle Tr-DNA.

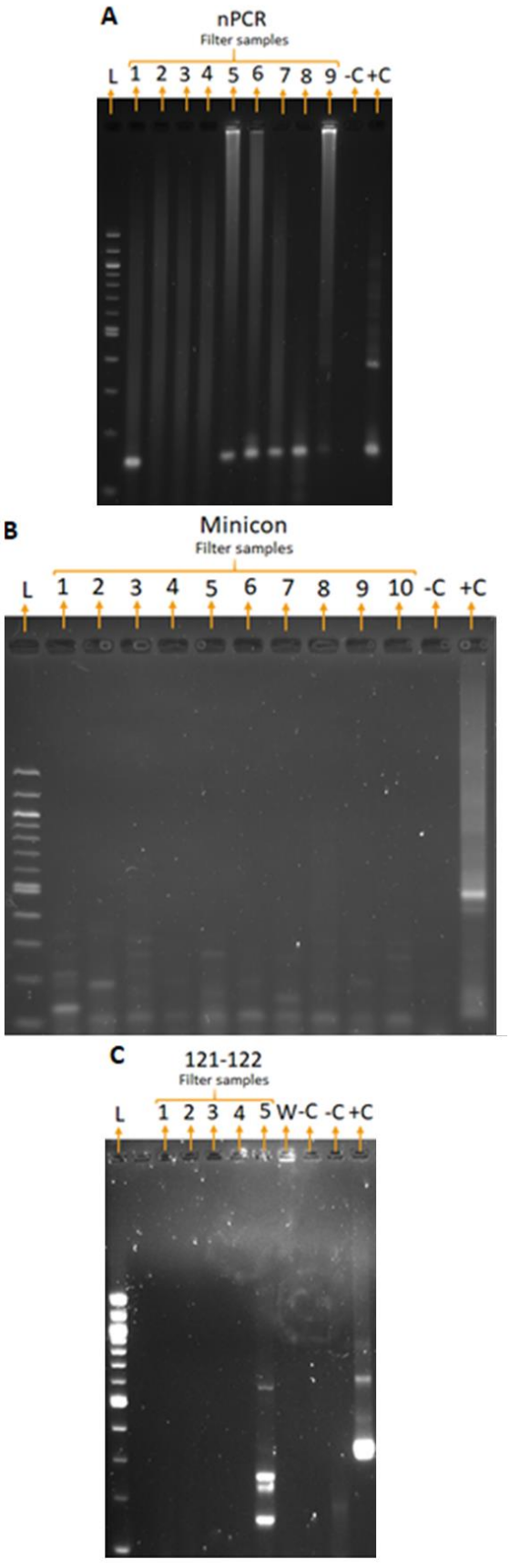

Figure 17) 2% agarose gel of primers: TCZ1-TCZ4 and Minicon-1-2:

A. nPCR, 149 bp

B. Minicon-1-2, 113 bp

C. 121-122, 330bp.

L= 100 bp Ladder, W=water control, A-E = urine filtered samples, -C= negative control, +C= positive control (gDNA). Each run was run blindly to prevent bias (sample numbers 1-10 are random).

PCR Analysis of Tr-DNA from the DC Cohort. Of the 36 urine samples that were tested using nPCR, Minicon and 121-122 primer sets, 13 were positive for *T. cruzi* Tr-DNA with at least one of the primer pairs. Of the 36 samples, seven were positive with nPCR, nine with Minicon, and only one with 121-122 (**Figure 18**-A). The 121-122 primer data was excluded for further analyses since it only contained one positive sample. The results from all of the samples can be found in Table 10 in the Appendix.

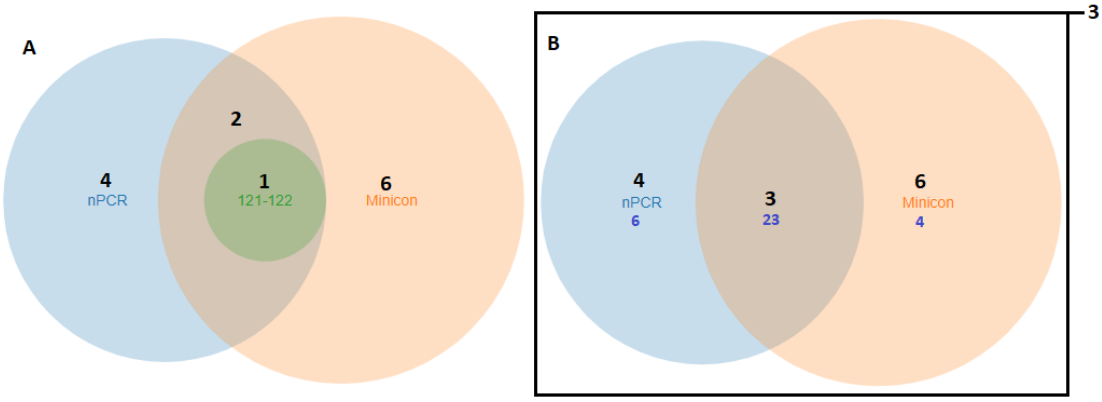

Figure 18) Venn Diagrams of nPCR, Minicon, and 121-122 **A.** summary of concordant positive samples distributing between nPCR, Minicon, and 121-122 in a series of 36 samples. **B.** Diagram summarizing concordant pairs between nPCR and Minicon, black color represents positive samples, and blue represents negative samples.

In **Figure 18**-B, the concordance between the nPCR and Minicon primers is visually represented as a Venn diagram. We observed that three samples were concordantly positive for *T. cruzi* Tr-DNA with both the nPCR and Minicon primer sets, while 23 samples were concordantly negative for all three primer sets (represented by the blue numbers in the diagram). A summary of the positive samples can be seen in **Table 7**.

Positive PCR			
Sample ID	nPCR	Minicon	121-122
229	1	1	1
359	1	1	0
361	1	1	0
218	1	0	0
208	1	0	0
358	1	0	0
357	1	0	0
327	0	1	0
345	0	1	0
216	0	1	0
360	0	1	0
380	0	1	0
274	0	1	0

Table 7) Table of positive sample results for nPCR, Minicon, and 121-122 (1=positive, 0=negative).

Only one sample (sample 229, **Table 7**) was positive for all three PCR primers. Between both nPCR and Minicon there were 13 PCR positive samples. Of this 13, only three were concordantly positive between both primers; six were positive with only Minicon, and four were positive with only nPCR.

Four participants (207, 219, 229, 388) provided an additional urine sample on a subsequent visit to the clinic. Each of these repeat samples (R) were analyzed and the results were compared to the results of the initial sample (I) (**Table 8**). The three participants who were negative for *T. cruzi* Tr-DNA with all three primer sets at the initial visit were also negative at the second visit. However, the repeat sample provided by one participant (sample 229 whose initial sample was positive for all primer sets) was positive for the nPCR primers, inconclusive for the Minicon primers and negative for the 121-122 primers.

Sample ID		nPCR	Minicon	121-122
I	207	-	-	-
R	207	-	-	-
I	219	-	-	-
R	219	-	-	-
I	229	+	+	+
R	229	+	#	-
I	388	-	-	-
R	388	-	-	-

Table 8) Analysis of replicate samples from four patients using the nPCR, Minicon and 121-122 primer sets. (# =inconclusive, + =positive, - =negative).

Serology. Of the 36 individuals who provided a sample, 34 were tested for *T. cruzi* antigens by serology. Two patients declined to provide a serum sample. For the serological analysis, samples were considered positive when two of the four tests results were positive (following Gilman lab's parameters). Of the 34 samples, 16 were designated to be serologically positive and 18 were negative.

Table 9 summarizes the difference in results observed between the serological tests used.

RT	
Positive	21
Negative	12
NA	1
Hemagen	
Positive	17
Negative	14
Indeterminate	3
Wiener Recombinant	
Positive	14
Negative	20
Wiener Lysate	
Positive	11
Negative	10
NA	13
TESA-blot	
Positive	14
Negative	16
NA	4

2 tests = final positive

Serology Final	
16	Positive
18	Negative

Table 9) Serology results summary in study population (n=34). RT= CDP whole blood Rapid Test.

PCR and Serology comparison. Of the 34 samples analyzed for Tr-DNA using nPCR and Minicon primers and for anti-*T. cruzi* antibodies, only one sample was positive for both Tr-DNA and antibodies (**Figure 19**). Serology was positive for 16/34 samples. For the seven nPCR-positive samples, three were concordantly positive with serology, and of the eight Minicon- positive samples, two were concordantly positive with serology.

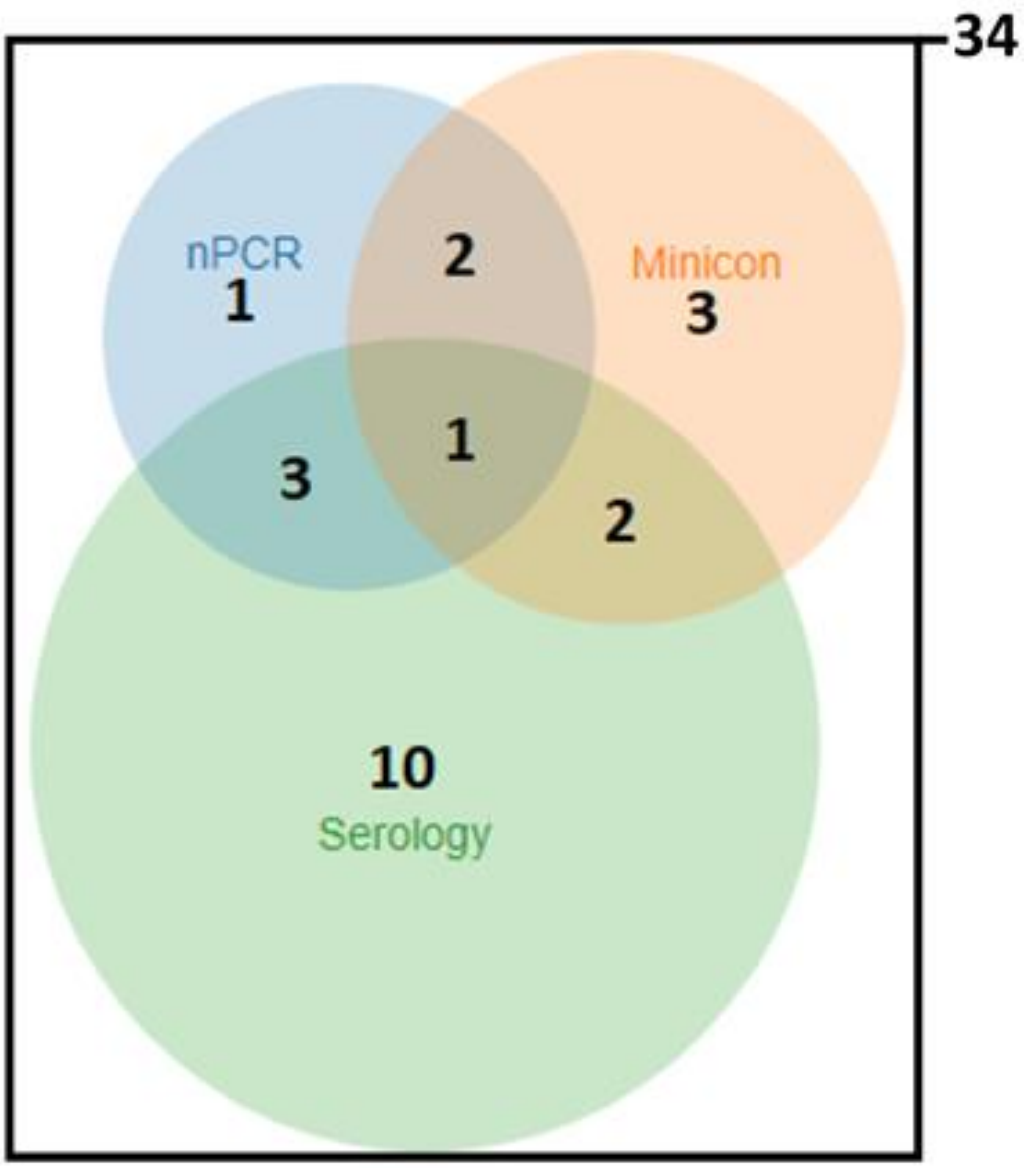

Figure 19) Venn Diagrams of PCR and Serology: summary of concordant pairs between nPCR, Minicon, and Serology final results.

Concentration Statistical Analysis. From the 40 urine samples collected, 38 were analyzed to test whether the volume of urine samples correlated with final concentration of DNA extracted. Two samples were omitted from this analysis as outliers from concentration and volume analysis, since their DNA concentrations were too high (80.8 and 71.1 ng/µL) due to menstruation/infection at time of collection Interestingly, this did not seem to affect PCR results from these samples. The average urine volume collected was 45 mL and the average concentration of DNA extracted from the filters was 2 ng/µL. No correlation was found between volume of urine filtered and DNA concentration extracted (p-value of 0.9253) (**Figure 20**). These results may be biased by amount of DNA a filter can capture and hold and the small number of samples.

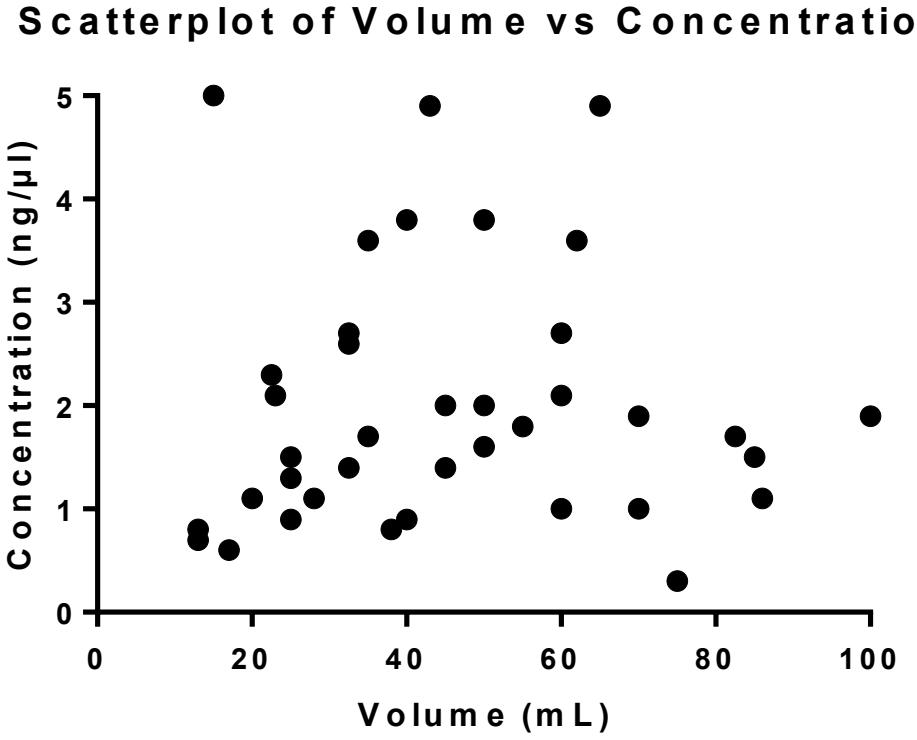

Figure 20) Correlation Scatterplot of volume vs concentration.

Discussion

Chagas disease has been a public health concern for a long time in Latin America. In recent years this concern has become more global with approximately 8 million people infected worldwide and at least 100 million at risk of infection[1,31]. However, this is likely an underestimate since these infections are difficult to detect and in non-endemic countries the index of underdiagnosis is estimated to be 95%[5,57]. This is due to a combination of asymptomatic cases, congenital infections, undocumented migrants with difficulty accessing care, and difficulty of diagnosis. Current diagnosis of Chagas disease is not straightforward, especially when it comes to the chronic phase of infection. Improving diagnosis to a direct method would help with faster and broader detection. In this study, we were able to detect *T. cruzi* DNA from patient's urine samples utilizing Tr-DNA PCR tests, which could provide a more accurate and less invasive method of detection in the future.

The validity of tests for Chagas disease depends on the phase of infection. In the acute phase (6-8 weeks) trypomastigotes are found in the blood (and, occasionally, other body fluids such as cerebrospinal fluid) and can be detected through direct microscopic visualization[5]. Following the acute phase, 30% of the patients transition into the chronic phase of infection, which can last 10-30 years[5], where amastigotes are intracellular and parasitemia is low and intermittent, making diagnosis difficult. Chronic phase diagnosis is not straightforward due to the intracellular nature of the amastigote stage, and tests to detect this phase rely on patient's history and clinical findings (previously diagnosed with acute infection, presentation with an enlarged heart with Chagas etiology, lived in Chagas

endemic country, mother previously diagnosed with Chagas) and indirect serological tests based on parasite-specific IgG antibody detection[59]. While there is no "golden standard" set up as reference, a confirmatory diagnosis requires two different serological tests, with a third test for confirmation when results are incongruous (that is after also including ECG's, X-rays, endoscopy, and other tests to observe disease progression when infection is suspected[57]).

All these tests require trained lab technicians and physicians in order to recognize and determine a confirmatory diagnosis. Additionally, serological tests have a highly variable sensitivity for samples obtained from different geographical regions[63,4,64], further complicating diagnosis. Better diagnosis tests are also needed in order to monitor treatment efficacy (when testing new antitrypanosomal drugs) since both drugs currently available for treatment are not optimal and present many adverse effects[76]. With serological tests, monitoring is not possible since they cannot discriminate between an active infection or successful cure post-treatment [5,,62]. They can give false positive results due to antibody persistence after the parasite has been cleared from the body. Serology can also provide false negative results. Treatment with current drugs, which only attack extracellular trypomastigotes[79,80], does not guaranty 100% parasite clearance and a patient with low level infection (intracellular amastigotes) may have anti-*T. cruzi* antibody titers too low to detect using the current tests.

To circumvent the issues with serological detecting and to improve diagnosis, many cf-DNA detection methods have arisen. Most focusing in PCR detection of *T. cruzi* from serum samples[7–10,68]. These PCR molecular techniques have proven to be highly specific and reproducible (targeting either sat-DNA or k-DNA), and are able to show positive

results days to weeks before trypomastigotes are visible by microscopy of peripheral blood[49]. However, so far, these methods are not sufficiently standardized to be used commercially and are only used in cases of inconclusive serological results [5,64,69,70]. They also focus on detection of DNA in serum, which is most useful during the acute phase of infection.

In order to be able to detect both acute and chronic infections, we have focused in detection of Tr-DNA from urine samples. Previous work performed in our lab has proven this method to be sufficiently specific and sensitive to be used as a diagnostic for other parasitic diseases such as *Schistosoma haematobium*[73], *Strongyloides stercoralis*[83], and *Taenia solium*[84]; being sufficiently sensitive to detect low and cryptic infections[83]. Not only that but the use of filtered paper to store DNA from urine samples proved to be cost effective, less time consuming, and facilitated specimen collection and transport. Other studies have also found cf-DNA in urine to be a useful biomarker for diagnosis of other parasitic diseases including malaria[62,85–87], African sleeping sickness[88], leishmania[89,90], toxoplasma[91]. With this large range of parasites where cf-DNA has shown to be a useful diagnostic, detection of pathogen-specific DNA fragments in the host urine can be argued to provide direct evidence that the pathogen is present[83]. Urine cf-DNA could also be used for treatment efficacy studies since, unlike antibody levels that remain high long after drug-mediated clearance of the pathogen, cell-free DNA is removed rapidly from the system as demonstrated by Ibironke et al. when examining schistosome infected cases two weeks post-treatment with praziquantel[92].

To determine whether *T. cruzi* Tr-DNA could be detected from patient's urine samples, samples were collected/processed/and tested with three different conventional PCR

primers targeting *T. cruzi* specific repeat regions (sat-DNA and k-DNA) and compared against serological results. For this study, 36 volunteers (18-60 years old) from a Chagas Disease cohort study in the DC-Virginia area provided urine samples, with four providing extra specimens for a total of 40 samples. All of the urine samples were processed through filters, and DNA was extracted using a commercial DNA extraction kit. PCR analysis followed, with two reference primer sets (121-122 targeting the kinetoplast variable region, and TCZ-1-4 targeting the sat-DNA conserved repeat) and one in house primer set (Minicon1-2 targeting the kinetoplast conserved domain) – additionally each sample was analyzed by the Gilman group using four serological tests per sample. For the purpose of this study, the presence of *T. cruzi*-specific DNA in PCR results was considered indicative of infection only after sequencing confirmation; and a sample was considered serologically positive after two positive serological results.

Of the 36 samples, we identified 13 positive for infection with PCR and 16 with serology (Figure 19 and Table 10). PCR positive fragments were identified in specimens with both positive and negative serological results. Of the 16 serologically positive samples, 6 were positive for parasite-derived DNA. The remaining 10 samples with positive serology results were negative by PCR analysis despite repetition, which might be suggestive of false positive serology results due to presence of anti-*T. cruzi* antibodies after disease clearance. Alternatively, it is possible that there was no observable DNA in the urine at time of collection or that *T. cruzi* DNA fragments other than the ones our primers amplified were present. Six samples were negative by serology and positive by PCR, which strongly suggests that the serology resulted in a high level of false negative findings. Presumably, while there are parasites present, there was not sufficient anti-parasite

antibody present in the blood when tested. This could be due to chronic disease with low antibody titer, or due to the presence of anti-parasite antibodies that recognized epitopes not present in the antigen preparations used to construct the five antibody-detection tests used in this study.

In the PCR gel electrophoresis results, it is also important to note that the positive controls used were *T. cruzi* genomic DNA, while sample DNA was Tr-DNA that most likely originated from dead and dying parasite cells, passive release, or active secretion[62,72,93]. While all 13 PCR positive samples were confirmed through sequencing, there were also differences in results observed depending on which PCR primer set used (See Table 5 for primers). Seven samples were positive with the nPCR primer set, nine with Minicon, and one with 121-122, not all of which were congruent with each other (See Figure 18 for PCR results). The nPCR, targeting sat-DNA TCZ minisatellite region, amplified the expected fragment of 149 bp for both samples and positive controls, as well as demonstrating brighter bands (higher sensitivity) than other primer sets used. This increase in sensitivity is attributed to the nested PCR protocol followed[82], in contrast to the conventional PCR method used for the other primers. The Minicon primer set, targeting the kinetoplast conserved domain segment, demonstrated the expected band of 113 bp in the positive control and samples as well as two differently sized bands at ~120 bp and ~300 bp in the samples. Similarly, primer set 121-122, targeting the k-DNA variable repeat domain, amplified the expected 330 bp fragment for the positive control, while amplifying 120 bp and 270 bp bands in different samples. The differently sized bands were demonstrated to have originated from *T. cruzi* k-DNA after sequencing, with blast hits of 95% identity to *T. cruzi* repeat segments.

The incongruent results between primer sets, as well as unexpected band sizes observed in samples, may be due to composition of the DNA (nuclear + kinetoplast) that is known to differ between DTU's due to the different number of repeat fragments[16] and/or variation in the sequence and size of the repeats. In addition, differences in band sizes between samples and positive controls may be due to processing and fragmentation of cf-DNA as it passes through the kidney to become Tr-DNA[71,72].

In order to gain insight into the origin of these unanticipated PCR products, we analyzed the sequencing results for each individual band. While the bands confirmed as *T. cruzi*-DNA fragments, the resulting sequences were larger than the predicted size. This size differences were likely due to amplification artifacts that happened during amplification or template generation, were extra sequence was added to the core *T. cruzi* repeat sequence. While a larger sample size would be needed to determine how the different size fragments were generated, these results confirmed that all of the PCR products originated from *T. cruzi* repeat DNA.

We also observed that the concentration of DNA for each sample did not correlate with the total volume of urine filtered (p-value= 0.9567, Figure 20). This is important to note since samples are collected in the field and the volume of urine collected from patients cannot be controlled. These findings would need further analyses with a larger sample size since results may be biased by the amount of DNA a filter can hold, and by how much of the filter is in contact with the urine. DNA urine concentration has also been shown to vary depending on time of day collected, diet, changing pH levels, storage, and extraction methods used[94–96]. These measurements only represent the total DNA extracted from each sample, since a nanodrop does not differentiate between human and parasite DNA we

cannot know what portion of the 2 ng/μL is human and what portion is *T. cruzi* DNA. We also noted that frozen urine samples (at -80^{o}C, for 1 year) were not optimal for PCR DNA analysis, since all DNA seemed to have degraded. While a bigger sample pool would be needed in order to be confirmed (for filtered urine method) our results agree with Soto et al.'s results[97], where they found that long-term frozen urine samples are not optimal for DNA extraction and use as template in PCR detection, regardless of extraction methods used.

V. Conclusion and Future Work

In this study we were able to demonstrate that *T. cruzi* DNA can indeed be detected from human filtered urine samples. While future work is still needed, these findings present the possibility for a non-invasive Chagas disease diagnostic test, with the possibility of accurately detecting both acute and chronic phases of the disease. In addition, this test could be used for early detection of congenital disease, disease re-emergence, and the monitoring of treatment efficacy.

Future Directions. *T. cruzi* Tr-DNA detection may be a more direct and specific measure of both the acute and chronic phases of Chagas disease. However, a larger number of samples would be needed in order to obtain its sensitivity when compared with current diagnostic methods. Primers may also need to be improved in order to recognize different DTU's and other repeat fragments found in urine samples not amplified with the primers used in this study. For this to be possible, Next Generation sequencing could be used to survey all of the parasite-derived DNA that is present in the urine from infected individuals. Sequencing urine samples through this method could identify additional *T. cruzi* repeat fragments present in the urine. This would lead to better primer design and the development of a multiplex-PCR system could provide a more sensitive and less variable method of diagnosis.

In order to improve current Chagas disease diagnosis, and decrease under diagnosis levels in non-endemic countries, I believe a new plan of action is needed. A compromise between serology and PCR analysis (one serology test and one PCR test, instead of two serological tests with a third as confirmation), in a point of care system, could be made in order to give faster confirmatory diagnostics. Furthermore, if an alternate parasite-detection method could be obtained from filtered urine samples, (for example detect both parasite antigens and Tr-DNA), this would further facilitate precision in the diagnosis of *T. cruzi* infections in humans.

Appendix

Supplementary Results - Sequencing

Sequencing + Bioinformatics. Bands were individually extracted and sent to Macrogen for sequencing, including differently sized bands found in samples. However, the resulting sequences obtained after sequencing did not agree with the size band that had been extracted from the gel (e.g. a band sized 149 bp would be extracted and sequencing results would return a 200-1300 bp fasta sequence) due to restrictions in sequencing parameters. In order to verify both the presence of DNA and confirm band size after extraction, all sample bands were then also run through a tape station. The results for the nPCR samples can be seen in **Figure 21**. The aggregated peaks at 149 bp represent the resulting band size, confirming that the bands extracted and sent for sequencing were this size. This process was followed for each positive sample band, for all sets of primers.

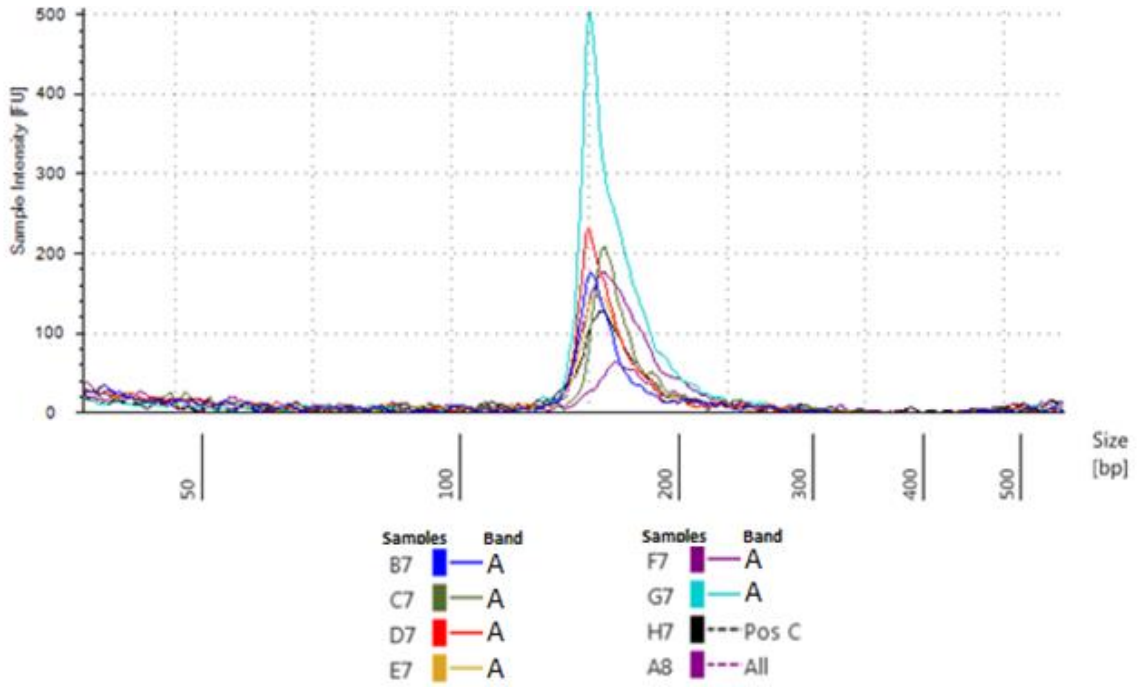

Figure 21) Tape station results. X=band size (bp), Y=Sample Identity. Each sample is individually indicated by a different color. Sample A8 is a pool of all samples, and H7 is the genomic positive control.

All presumptive positive samples were also confirmed as *T. cruzi* positive through BLAST after sequencing and tape station size confirmation. **Figure 22** demonstrates the Blast results for one sample. As mentioned above, results from sequencing were longer than expected for each sample (in this case a positive sample band from nPCR of 149 bp was sent and a 700 bp sequence was returned, even after being confirmed to be 149 bp by tape station analysis).

Figure 22) NCBI BLAST results. In blue is the query sample sequence, and smaller pink lines all represent hits to T. cruzi Sat-DNA. On the right are results for the first three hits.

These results not only re-confirmed the expected band sizes, they also allowed for query sample sequences to be cleaned, and a sample library was formed for each primer set. As well as confirming that the bands observed were *T. cruzi* positive (including differently sized bands observed with primer sets Minicon 1-2 and 121-122). These sample results were also compared against the sequence obtained from the positive control for further confirmation (**Figure 23**).

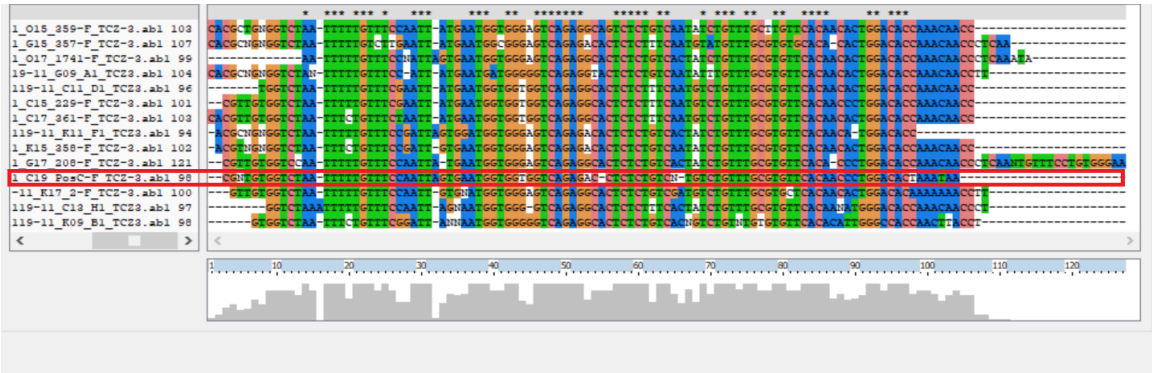

Figure 23) Sample alignment against positive control. ClustalX results demonstrating alignment with asterisk (*) representing amino acid placement conserved through all sequences. Positive control is signaled by the red square.

Through these analyses we were able to also observe the repetitive nature of this small DNA fragments (**Figure 24**). While not all hits were repeats, and there was much variability between results, all sample bands run for the different primer sets were confirmed to be part of tandem repeats. While further analysis and more samples would be needed to demonstrate that the differently sized bands are fragments from these tandem repeats, these results are presumptive evidence.

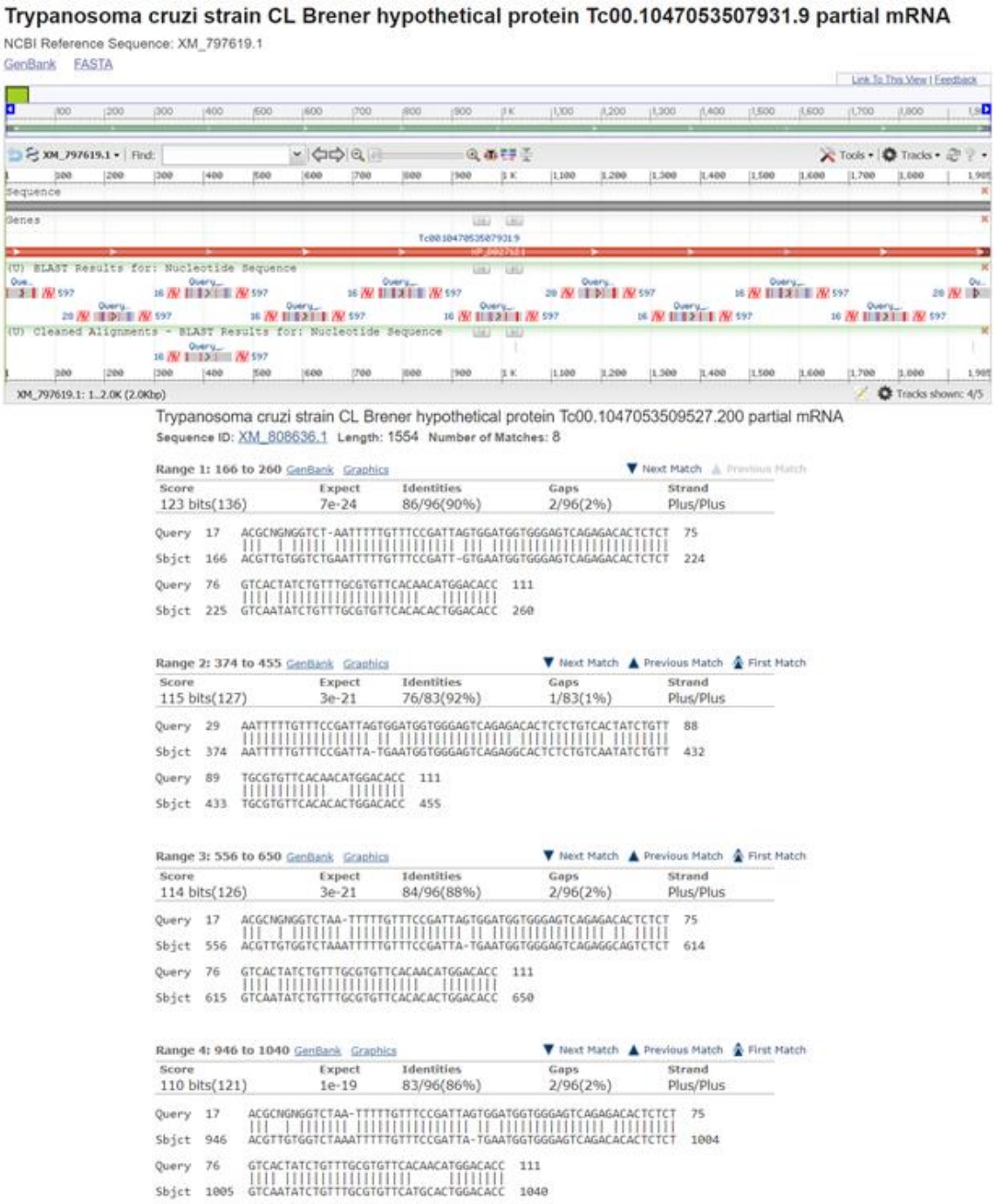

Figure 24) NCBI BLAST repeat results. Positive sample sequence was used as query sequence. It is observed to be repeated 12 times with different hits to a T. cruzi hypothetical gene.

A. IRB Approval

JOHNS HOPKINS
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FWA #00000287

Institutional Review Board Office

615 N. Wolfe Street / Room E1100
Baltimore, Maryland 21205-2179
Phone: 410-955-3193
Toll Free: 1-888-262-3242
Fax: 410-502-0584
Email: jhsph.irboffice@jhu.edu
Website: www.jhsph.edu/irb

AMENDMENT APPROVAL NOTICE
EXPEDITED REVIEW

Date: March 13, 2018

To: Robert Gilman, MD
Department of International Health

From: Joann Katz, ScD
Chair, IRB-FC

Re: **Study Title**: "Seroprevalence and Characterization of Chagas Disease Cardiomyopathy among Latin Americans Living in Fairfax County, VA"
IRB No: 00006713

The JHSPH IRB received the amendment request described below on February 28, 2018.

The IRB reviewed and approved this request on **March 5, 2018.**

This amendment approval is:

1. To add Clive Shiff and Alan Scott as co-investigators to the study team.
2. To add Gaby Madrigal and Kelly DeToy as student investigators to the study team.

As a reminder, no other changes to this study may be implemented without prior JHSPH IRB review and approval.

The action taken on this study does not change the IRB expiration date, which remains **October 31, 2018.**

If you have any questions regarding this action, please contact the JHSPH IRB Office at (410) 955-3193 or via email at jhsph.irboffice@jhu.edu.

JK:lm

B. Argentina preliminary sample (F) vs. DC samples with primer 121-122.

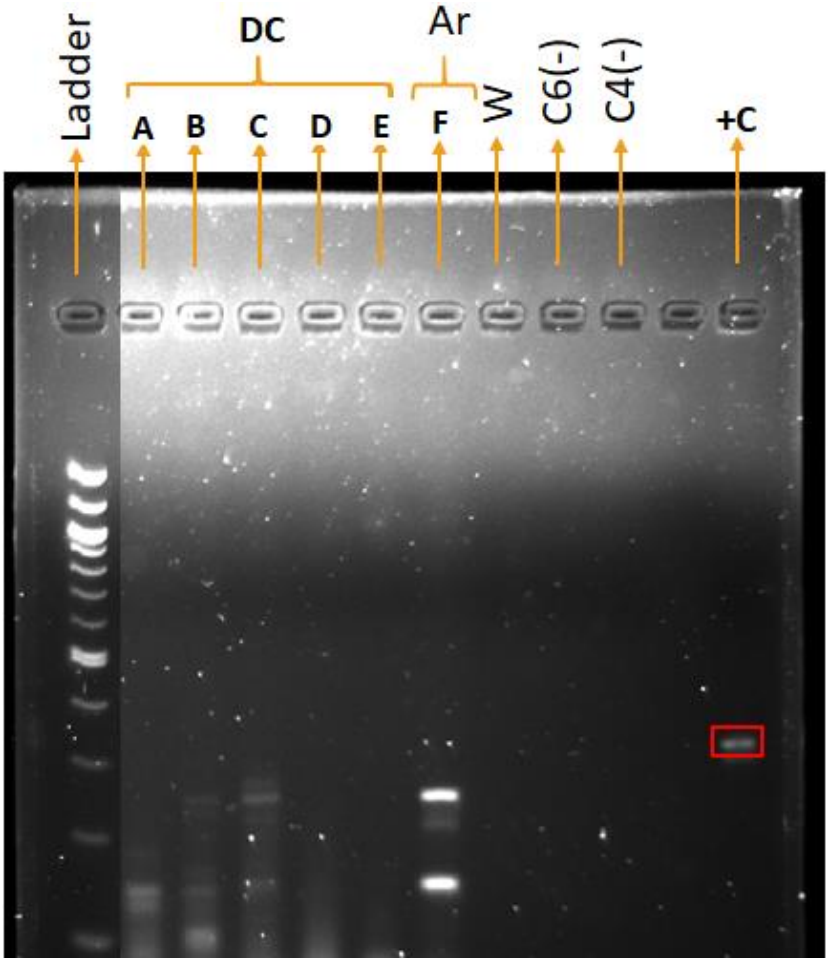

Figure 25) DC samples vs Argentina sample. 2% agarose gel of primers 121-122 (330bp) L= 100 bp Ladder, W=water control, D-E = urine filtered samples, -C= negative control, +C= positive.

C. Results Master Table.

1	2	3	PCR			Serology						13	14	15
			4	5	6	7	8	Wiener recombinant 9	10	11	12			
ID	Volume (mL)	Con. (ng/µl)	nPCR	Minicon	121-122	Rapid Test	Hemagen		Wiener lysate	TESA-blot	Final	Sex	Origin	Age
229-I	35	1.7	1	1	1	0	1	0	1	1	1	F	(9) El Salvador	43
359	15	5	1	1	0	0	0	0	0	0	0	F	(1) Argentina	45
361	82.5	1.7	1	1	0	0	0	0	0	0	0	F	(3) Bolivia	38
218	85	1.5	1	0	0	1	2	0	NA	0	1	M	(3) Bolivia	34
208	23	2.1	1	0	0	1					0	M	(9) El Salvador	63
358	62	3.6	1	0	0	0	0	0	0	0	0	F	(1) Argentina	45
357	43	4.9	1	0	0	1	1	1	1	1	1	M	(3) Bolivia	48
327	25	1.5	0	1	0	0	1	0	1	1	1	F	(3) Bolivia	55
345 (fung	55	80.8	0	1	0	NA	0	0	0	0	0	F	(3) Bolivia	42
229-R	32.5	2.6	0	1	0	0	1	0	1	1	1	F	(9) El Salvador	43
216	50	3.8	0	1	0	1	0	0	0	0	0	F	(10) Guatemala	27
360	40	3.8	0	1	0	0	0	0	0	0	0	F	(3) Bolivia	46
380	32.5	1.4	0	1	0	0	0	0	NA	0	0	F	(9) El Salvador	28
274 (fung	35	71.7	0	1	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
356	22.5	2.3	0	0	0	1	1	1	1	1	1	F	(3) Bolivia	57
324	25	0.9	0	0	0	1	1	1	1	1	1	F	(9) El Salvador	27
355	45	2	0	0	0	1	1	1	1	1	1	M	(9) El Salvador	66
388-I	45	1.4	0	0	0	1	1	1	1	1	1	M	(9) El Salvador	49
388-R	38	0.8	0	0	0	1	1	1	1	1	1	M	(9) El Salvador	49
234	28	1.1	0	0	0	1	1	1	NA	1	1	F	(9) El Salvador	35
435	40	0.9	0	0	0	1	1	1	NA	1	1	F	(3) Bolivia	29
141	86	1.1	0	0	0	1	1	1	NA	NA	1	M	(3) Bolivia	44
261	100	1.9	0	0	0	1	1	1	1	NA	1	F	(3) Bolivia	52
180	20	1.1	0	0	0	1	1	1	1	1	1	F	(9) El Salvador	40
109	13	0.8	0	0	0	1	1	0	NA	1	1	F	(9) El Salvador	48
129	70	1	0	0	0	1	1	1	1	1	1	F	(9) El Salvador	52
183	17	0.6	0	0	0	1	1	1	1	1	1	F	(9) El Salvador	41
163	13	0.7	0	0	0	1	0	0	0	0	0	F	(9) El Salvador	51
413	55	1.8	0	0	0	1	0	0	NA	0	0	F	(9) El Salvador	26
219-I	50	2	0	0	0	1	1	1	NA	1	0	NA	(10) Guatemala	NA
219-R	35	3.6	0	0	0	1	2	1	0	0	0	NA	(10) Guatemala	NA
337	75	0.3	0	0	0	1	0	0	NA	0	0	M	(7) Costa Rica	48
207-I	65	4.9	0	0	0	1	1	0	0	0	0	F	(9) El Salvador	19
207-R	50	1.6	0	0		1	1	0	0	0	0	F	(9) El Salvador	19
321	32.5	2.7	0	0	0	0	0	0	0	0	0	F	(16) Peru	36
214	70	1.9	0	0	0	0	2	0	NA	0	0	F	(9) El Salvador	28
237	60	2.7	0	0	0	0	0	0	NA	NA	0	M	(9) El Salvador	49
200	60	2.1	0	0	0	NA	NA	NA	NA	NA	0	F	(3) Bolivia	NA
759	60	1	0	0	0	0	0	0	NA	0	0	M	(9) El Salvador	62
133	25	1.3	0	0	0	0	0	0	NA	NA	0	F	(9) El Salvador	30

Table 10) Results master table. In the first column are the sample identification numbers (ID), highlighted in in blue are the repeated samples. Second column is urine volume of specimen in mL and third column is DNA concentration extracted in ng/µL. Fourth to sixth column are PCR results, and Seventh to twelfth column are serology results; positive results are represented by a "1" and highlighted in yellow, negative results are represented by a "0", inconclusive results are represented by a "2", and NA represents no sample available. In the thirteenth column "F" =Female and is highlighted in pink, "M" =Male and is highlighted in blue. The fourteenth column shows patient origin with different geographical areas represented by different numbers and text color. Finally, the fifteenth column shows the patient's age. In the three last columns "NA" =no answer.

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CV

Ana Gabriela Madrigal, Sc.M.

Home Address:
3056 Adams Ranch Ct.
Chula Vista, CA 91914
Cell: (619)247-1288
Email: agmadrigal2@gmail.com

Education

Expected May 2019 — **Masters of Science (Sc.M.)**
Department of Molecular Microbiology and Immunology
Johns Hopkins Bloomberg School of Public Health
Master's Thesis: Chagas Disease, a Diagnostic Enigma
Related coursework: Immunology, Parasitology, Vector Biology, Virology, Statistical Computing

December 2015 — **Bachelor of Science (BS)**
Major: Biological Sciences: Emphasis Microbiology and Immunology
Minor: Chemistry, Cognitive Science
University of California Merced
Related coursework: Biochemistry, Parasitology, Neuroscience, Neurobiology, Virology, Cancer Genetics & Tumor Biology, Inorganic Chemistry, Organic Chemistry, Scientific Computation for Chemistry

Research Experience

Nov 2017-Present — **Graduate Researcher** Shiff Lab, Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health
Research project: Diagnosis of Chagas Disease by Detection of *Trypanosoma cruzi* DNA Fragments from Filtered Urine Samples

- Develop and optimize primers and their PCR protocols
- Optimize extraction techniques for filtered urine, as well as perform extractions from blood and unfiltered urine using kits
- Perform data analysis and generate tables and figures
- Utilize Nanodrop, FluorChem imagers, and sequencing programs
- Collaborate on drafting IRBs and building consent forms for human sample collection
- Train new lab members in PCR and gel electrophoresis techniques and oversee lab management
- Developed a project and new lab protocols for lab high school trainee

Feb 2016- Aug 2017 — **Graduate Researcher** Saier lab, Section of Molecular Biology, University of California San Diego
Research project: Bioinformatic Characterization of the YedE/YeeE Family of Putative Membrane Transporter Proteins

- Homologue identification through PSI-BLAST and CD-HIT
- Protein classification utilizing multiple sequence alignments
- Defined functional motifs with MEME
- Analyzed protein topology with HMMTOP
- Performed Phylogenetic analysis using an in-house program called SuperFamilyTree

Oct 2016-Aug 2017 **Graduate Researcher** Caffrey lab, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego

- Cultured *Trypanosoma brucei brucei*
  - Ran high throughput assays and analysis
  - Including: passaging, screening, plate reading, and data analysis
- Cultured *Caenorhabditis elegans*
  - Assisted the development of an automated protocol

Aug 2013-Aug 2015 **Undergraduate Researcher** Hirst lab, Soft Matter and biophysics, University of California Merced

- Focused on the structure and phase behavior of lipid membranes
- Developed lipid vesicles utilizing DOPC and DPPC lipids
- Analyzed vesicle mechanical properties using a fluorescent microscope and dual beam optical laser trap

Teaching Experience

Nov 2017-Present **Lab Mentor**, Shiff Lab, Department of Molecular Microbiology and Immunology,

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

- Oversee training and supervision of high school student for the Ingenuity Science programs at the Baltimore Polytechnic Institute
- Instruct student on the scientific technique, PCR and extraction protocols

Oct 2018 - Dec 2018 **Teaching Assistant,** Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD
Course Title: Biology of Parasitism

- Maintained Lab Notebook Guide, organized microscope slides for lab sections, communicated with students, held office hours, and graded assignments. 1 term (Dr. Clive J. Shiff & Dr. David A. Sullivan)

Leadership Experience

2013 - 2014 **Vice President of Hip Hop Movement**, University of California Merced
[HHM is a nonprofit organization that combines the four branches of Hip Hop: DJ, Graffiti, Spoken Word, and Dance. It strives to bring together the community and
the student population to teach the culture of Hip Hop]

- Managed paperwork for on and off-campus events
- Networked with several organizations
- Coordinated a series of events with other team members, organizing competitions and headliner
- Participated in student assembly discussions
- Assisted in the initial development of the first Black Arts Movement Conference held at UCM
- Managed what totals to $10,500 in events and raised funds for the club

Skills and Additional Training

Professional Training

Expected 2019	Certificate in Tropical Medicine, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD
Oct 2017	Biosafety: Bloodborne Pathogens Training, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego
Feb 2016	Annual Laboratory Hazards Training, Section of Molecular Biology, University of California San Diego
Feb 2016	UC Laboratory Safety Fundamentals, Section of Molecular Biology, University of California San Diego
2015-2016	Certificate in CPR/AED, University of California Merced

Software

- Proficient with Excel, Word, PowerPoint and other Microsoft Office Software
- GraphPad Prism
- Bioinformatic tools: BioV Suite, Fig Tree, MEME
- Alpha View
- PSI-BLAST, Primer-BLAST (NCBI)

Programming

- Basic scripting knowledge in R, BASH Shell, Linux, Python, C, and C++

Technical Skills

- FluorChem imagers
- High Throughput analysis
- PCR
- ELISA
- Cell Culturing
- DNA Extractions
- Electrophoresis
- Sequence Analysis

Languages

- Fluent in English and Spanish